Maintenance of immune fitness during reconstitution from T cell lymphopenia by CD4-positive, CD25-positive, and Foxp3-positive regulatory T cells

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Dedication

This thesis is dedicated to my parents, Jean and Theodore Winstead, who taught me the value of integrity and hard work, and through much personal sacrifice made my higher education, this thesis, and my degree possible.

Abstract

Work presented in this doctoral thesis focuses on the role of regulatory T cells (Tregs) in controlling T cell homeostasis and emergence of autoimmunity during immune reconstitution from lymphopenia. It is recognized that lymphopenia may be a common trigger of many autoimmune diseases due to oligoclonal expansion of self-reactive T cells with an effector phenotype. It is also a clinical fact that autoimmunity is often associated with immune deficiency and poor responsiveness to vaccines and infections. Research supporting work presented in chapter 2 of this thesis was based on the hypothesis that poor Treg function may play a central role in these phenomena. This work, published in June of 2008 in the *Journal of Immunology*, clearly demonstrated that Tregs selectively restrain one specific form of lymphopenia-induced proliferation characterized by burst-like cell cycle activity and effector T cell differentiation (*spontaneous* proliferation). The spontaneous form of lymphopenia-induced proliferation is the likely source of oligoclonal expansion of self-reactive T cells that drive autoimmunity. Work presented in chapter 3 of this thesis addresses the hypothesis that such oligoclonal expansion by a few T cell clones consumes resources away from the rest of the T cell population, which ultimately results in loss of T cell diversity. Using the technique of T cell adoptive transfer, we measured immune responses to infection with the gram negative bacteria *Listeria monocytogenes* in lymphopenic mice reconstituted in the presence or absence of Tregs by analysis of T cell receptor (TCR) Vβ chain usage and repertoire sampling using Vβ-Jβ chain TCR spectratyping and magnetic bead enrichment with specific major histocompatibility (MHC) class I and II tetramers. Experimental results suggest that the presence of Tregs during immune

reconstitution preserves TCR structural diversity and allows for more accumulation of pathogen-associated antigen-specific T cells in secondary lymphoid tissues following clearance of the infection. This result may otherwise seem paradoxical as Tregs are typically thought of as generalized suppressors of the immune system. Regardless, we believe this unappreciated ability to maintain T cell homeostasis through preservation of peripheral diversity will shed considerable insight into the role of Tregs in the immune system, vaccine responsiveness, pathogenesis of autoimmunity, and immune senescence.

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Chapter 1

Introduction

Establishing a competent immune system: basic concepts and components

The purpose of the mammalian immune system, in simple terms, is to defend the host organism against pathogenic invaders while maintaining tolerance to self. A competent mammalian immune system is comprised of two basic branches: the innate and the adaptive. The innate branch of the immune system serves as first line of defense to pathogenic invasion, in that cellular components are sensitive and responsive to inflammation and infectious signals without the requirement for specificity. Innate immunity also primes the adaptive immune system, establishing specificity and memory essential to defense against future insult as well as prevent autoimmunity. Cellular components of innate immunity essential to coordination with adaptive immune cells include the antigen-presenting cells (APCs). Virtually all nucleated cells can function as antigen presenters, however APCs expressing proteins necessary for direct cell-to-cell interaction and activation of adaptive cells are termed 'professional' APC. The three major cell types meeting the requirements for this classification include macrophages, dendritic cells (DC), and B cells. Though macrophages and B cells play necessary roles in establishing adaptive immunity and express the necessary receptors for direct recognition of pathogen products, DCs are arguably the most important APCs (1). DC are exquisitely sensitive to inflammatory signals encountered at the site of pathogen invasion. As immature cells, they express a wide range of pathogen recognition receptors (PRRs), of which toll-like receptors (TLRs) are the best studied. DC biology is tightly controlled. Maturation in response to inflammatory signals at the site of pathogen invasion induces up-regulation of receptors necessary for migration to

lymphoid tissues and interaction with adaptive immune cells, and their ability to process and present antigens in the context of cytokine signals that direct the activation and differentiation of B and T cells (2).

Activation and differentiation of CD8-expressing cytotoxic T lymphocytes (CTLs) and CD4-expressing, T helper (Th)-type effector cells is implemented through three stimulatory signals provided by primed APC (3, 4). Signal one constitutes the ligation of the T cell receptor (TCR) with foreign peptide presented in the context of self-major histocompatibility complexes (MHC) on DCs. Generally, peptides generated from processing of intracellular pathogens, including viruses and some species of bacteria, are presented in the context of MHC class I and induce activation of CD8 T cells. Recognition of extracellular bacteria, fungi, and metazoans generally results in processing and presentation of peptides on MHC class II to CD4 T cells (5). Signal two constitutes up-regulated expression of co-stimulatory molecules on the surface of mature DCs (e.g. B7) and ligation of T cell co-stimulatory receptors (e.g. CD28) (6). The final signal provided by CTL differentiation and Th cell polarizing molecules is largely dependent on the conditions under which DCs have been primed (i.e. what the 'milieu' looks like to DC) (7-9). An emerging area of study regarding DC biology seeks to define requirements for TLR (inflammatory) and Notch (non-inflammatory) signaling, and resultant cytokine production, in regulation of T cell differentiation (10).

T Cell Homeostasis

Central tolerance is established in the thymus through the processes of positive and negative selection for T cells with weak recognition of self-peptide antigen in the context of self peptide-MHC. Mature T cells emigrate to the periphery where they retain enough self-reactivity for survival and basal proliferation, while at the same time exhibiting strong reactivity to foreign, pathogen-derived antigens.

Secondary to structural T cell diversity established in the thymus is the diversity determined through homeostasis in the periphery. T cell homeostasis in the periphery is determined over time and space by the niche, which is in turn determined largely by availability and recognition of two categories of cell-extrinsic signals: cognate antigen and homeostatic cytokines. Survival and basal proliferation signal requirements for a naïve T cell differ from those of a cell that has encountered its foreign antigen in the context of an antigen presenting cell (effector - T_{eff}, effector memory - T_{EM}, or central memory – T_{CM} cell) (11).

Peripheral homeostasis of naïve T cells, the magnitude of a T cell response to a particular pathogen, and the quality and quantity of memory established is regulated in large part by cytokine signals delivered through the γc chain cytokine receptors (IL-7, -2, and -15) (12). IL-7, a cytokine produced and constitutively presented by thymic and lymphoid tissue stroma, is essential to survival of naïve CD4 and CD8 T cells (13). Intermittent recognition of IL-7 in the periphery prevents naïve T cell apoptosis, stimulates basal cell cycling, and, in collaboration with self-p-MHC, establishes the peripheral, naïve T cell 'niche' (14, 15). Although T cells lose sensitivity to IL-7 upon

activation, it again becomes an important survival signal for memory T cells, in that they again up-regulate expression of the receptor. Memory T cells, in addition to IL-7, are also very sensitive to IL-15 signaling. Recognition of IL-15 trans-presented as a complex with the receptor α chain on the surface of bone-marrow-derived cells by T cells bearing the low affinity IL-2 receptor (IL-2Rβ:γc) plays an essential part in homeostasis of central memory CD8 T cells and may play a part in regulating pathogen-specific CD4 T cell responses. The role IL-2 plays in homeostasis of T cells is, as well as IL-15, highly complex and lacking in complete definition. Both cytokines share the same low-affinity receptor and both support memory T cell homeostasis, however IL-2 favors support of the effector memory T cell phenotype, by virtue of the fact that IL-2 is produced primarily by activated CD4 T cells. In addition, IL-2 plays a major part in maintenance of peripheral T cell tolerance through mediation of activation-induced cell death (AICD), a process whereby self-reactive cells are eliminated (16). Important aspects of peripheral tolerance will be discussed in more detail below.

T cell *structural* repertoire diversity

The first step in establishing T cell repertoire diversity takes place in the thymus during development (17). Here, a developing αβ T cell is selected for survival based on successful expression of a structural complex receptor on its surface that recognizes MHC presented by thymic epithelia. This receptor (TCR) is composed of an α and β chain encoded by several dozen variable, randomly arranged gene segments denoted as variable (V), diverse (D), and joining (J) (18). Specificity is conferred to the receptor

primarily through deletion and addition of nucleotides at the junctions between these fragments (complementarity determining region 3 or CDR3). Receptors that recognize self-peptides in the context of self-MHC weakly confer survival signals to the cell and allow maturation. The outcome of thymic selection is seeding of the periphery with strongly foreign antigen-reactive, self-tolerant T cells, each of which expresses ~20,000 receptors all with the same peptide antigen specificity. Estimates of αβ T cell receptor diversity based solely on potential gene segment combinations range between 10^{12} and 10^{14} (mouse and human). Restrictions imposed by thymic selection and peripheral survival signals reduce this estimate to roughly 10^{8}, or 0.000001% of the potential genetic combinations. This is likely a gross overestimate of the actual functional repertoire in any given individual, however, based on further requirements for affinity and avidity of TCRs for peripheral self-antigens supporting homeostasis (19).

T cell *functional* repertoire diversity

As already mentioned briefly above, naïve CD4 and CD8 T cells, upon antigen encounter in the presence of co-stimulation and cytokine recognition, undergo a process of activation and differentiation. The goal, and usual outcome, of this activity is maintenance of T cell repertoire fitness. Both CD4 and CD8 T cells adopt 'effector' phenotypes to facilitate clearance of a pathogen. Following pathogen clearance, many of the effector T cells die, and some effector cells transition to a memory phenotype. Central memory cells are thought to reside in the secondary lymphoid tissue (lymph nodes and spleen), whilst the effector memory population functions primarily in the

capacity of peripheral tissue immunosurveillance. The kinetics of each programming step differ for CD4 and CD8 T cells and are dictated by several complex factors, including the site and degree of infection, and by the biology of the infecting organism. Generally, CD8 T cells adopt a cytolytic phenotype for clearance of intracellular pathogens (viruses and bacteria). CD4 T cells, on the other hand, may adopt one of many defined phenotypes. In addition, CD4 T cells provide 'help' in the form of cell-surface and secreted signals to CD8 T cells and B cells as part of their activation program. CD4 T cells respond to most extra-cellular, bacterial pathogens by adopting a helper type 1 phenotype (Th1) and secreting the cytotoxic molecule interferon-gamma (IFN-γ). Most responses to fungal pathogens are thought to elicit differentiation of IL-17-secreting, Th17 CD4 T cells. This complex cytokine is thought to target endothelial and epithelial cells, as well as other T cells, and has been reported to both exacerbate and attenuate inflammation (20). Allergens are traditionally believed to stimulate a Th2 differentiation program in responding T cells, exemplified by production and secretion of interleukins 4, 5, and 13 that serve to recruit other innate immune cells to the site of exposure. What is perhaps the most complex phenotype CD4 T cells may adopt is one that functions to regulate other innate and adoptive immune responses. This particular CD4 T cell phenotype is a focus of work presented here and will be discussed in detail below.

Techniques to study T cell diversity

Studies focusing on T cell repertoire diversity include attempts to reduce this overwhelming complexity through manipulation of antigen receptor genes (21). The best examples are the generation of TCR Tg animals in which the majority of T cells harbor receptors with the same specificity, and the pairing of a TCRβ chain transgene with a TCRα minilocus comprised of a limited number of variable and junctional gene segments. These models of 'limited' diversity have been extremely useful for studies of both thymic selection and peripheral activation and differentiation (22). They are appealing in the sense that the cells retain diversity in their ability to differentiate while at the same time being easy to track over time. The modern technique of TCR CDR3 spectratyping was pioneered by Pannetier and colleagues in 1993 (23). This PCR (polymerase chain reaction)-based technique allows detection of patterns in the repertoire based on structural determination without the need for determinations of antigen specificity. One may, therefore, 'tackle' the issue of diversity without the need for transgenics. It also avoids the complexity involved with sequencing of the TCR (determinations of contact 'footprint' with MHC), which often require isolation of single cells by limiting dilution and amplification of template material through cloning. The transfer of immune cells from one animal to another, or 'adoptive transfer', as a concept was first introduced by Medawar in 1899 and refined by Mitchison in 1957 for improvements in engraftment (isogenic transfers into lymphopenic hosts) (24, 25). The technique has evolved over the years alongside methods for purification and sorting of donor tissue, and the generation of genetically altered mice allowing manipulation of

engraftment. This was especially useful for studies involving deconstruction of complex human immune diseases. Until the recent advent of MHC tetramer technology and the reliable detection of antigen-specific lymphocytes within unsorted populations, however, questions regarding repertoire diversity and precursor frequency of specific cells in wild-type, un-manipulated animals went largely unanswered (26-28).

The concept of immune cell diversity as a countermeasure against pathogen diversity

Much controversy exists regarding the role commensal bacterial and fungal antigens play in regulation and maintenance of mammalian peripheral T cell homeostasis. This controversy stems in part from the fact that tolerance is maintained to gut, skin, and other mucosal flora antigens in absence of expression in the thymus during negative selection of T cells. It is at present unknown how many, and to what extent, flora antigens cross-react to self-antigen-specific T cells that have undergone negative selection in the thymus, however published evidence suggests absence of flora antigens alone results in dysregulated T cell homeostasis (29).

The 'hygiene hypothesis' postulates that our chronic exposure to microbes, be they commensal or environmental, shapes and maintains the quality of our immune system (30, 31). Though conjectural, there is evidence to support this theory based on correlations of autoimmune disease and allergy in the western world with cleanliness of our living environment (32). Published evidence also suggests chronic helminth infections in mice and man are associated with protection from pathogenic microbial

and viral infection (33). On the other hand, exposure to viral pathogens early in life, such as those western world children are vaccinated against (e.g. measles), is positively correlated with inflammatory bowel disease (IBD) in adulthood. The Firmicutes and the Bacteroidetes are the predominant bacterial phyla colonizing the mammalian gut – with as many as 100 trillion microorganisms (34, 35). Data on the use of probiotics to treat gastrointestinal conditions is, as of yet, largely circumstantial. However, existing data suggests a potential prophylactic treatment for allergy is establishing or preventing disruption of enteric flora including these phyla and the other 4, relatively underrepresented phyla, known to colonize the human gut (36, 37). Much of the conjecture regarding the balance of commensal bacteria with healthy immunity is based on potential for differentiation of anti-inflammatory innate and adaptive cellular responses.

Peripheral T cell tolerance: the role of regulatory T cells

The processes of clonal deletion implemented by selection in the thymus and BM are imperfect. There must be additional peripheral mechanisms by which lymphocytes are rendered non-functional and tolerant to self and/or inappropriately MHC-presented antigens (38). Mechanisms of peripheral T cell tolerance are varied and complex, but can generally be categorized as ‘passive’ or ‘active’. Passive tolerance or suppression is based on competition between conventional cells for homeostatic signals (i.e. self p-MHC, costimulation, and cytokines like IL-7 and IL-15). Failure to receive context-dependent homeostatic signals in many cases results in anergy

(unresponsiveness) or apoptosis (cell death) (39). A third party mediates active tolerance either directly or indirectly. DC are an essential third party mediator to active tolerance, however the cell population perhaps best defined for its active tolerizing function is the $CD4^+Foxp3^+$ regulatory T cell (Treg).

Regulatory $CD4^+Foxp3^+$ T cells (Tregs) constitute 5-15% of peripheral CD4 T cells in healthy, adult mice and humans (40), and are functionally suppressive *in vitro* and *in vivo* toward a range of immunologic responses (41). Tregs require signaling through the co-stimulatory receptor CD28, the γc chain, and the β chain of the IL-2 receptor, as well as TCR engagement for development, function, and peripheral homeostasis (42-45). They are characterized by constitutive expression of IL-2Rα (CD25), the cytotoxic T lymphocyte antigen 4 (CTLA-4), the tumor necrosis factor (TNF)-receptor family members GITR (glucocorticoid-induced TNF receptor-related protein) and OX40, and the CD4 homolog LAG-3 (46, 47). The transcription factor Foxp3 is necessary and sufficient for Treg lineage commitment and suppressive function, and serves as their best phenotypic marker (48-50). Though our knowledge regarding thymic development is by no means complete, we can now link intracellular IL-2Rβ/signal transducer and activator of transcription 5 (STAT5) and TCR/nuclear factor-kB (NFkB) directly to expression of Foxp3 in precursor T cells (51).

The primary function of Tregs is to allow induction and resolution of host protective immune responses. These cells play a critical role in prevention of autoimmunity and possibly have therapeutic potential for established autoimmune disease (52). Treg deficiencies generally manifest in one of two ways: reduction in number or reduction in function.

Well-defined spontaneous Treg deficiency models include the *scurfy* mouse and the human counterpart: immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. In both cases, the deficiency in Tregs results from point mutations in the Foxp3 gene. Some other notable models well studied in mice include defects in expression of CTLA-4, CD28 (on T cells) and B7 (on DC), and CD25.

Controversy regarding Treg development: Thymus-derived (tTregs) vs. peripherally-induced (iTregs)

Like conventional CD4 T cells, Treg TCR diversity is established by selection in the thymus. As already discussed, negative selection (deletion) of conventional T cells occurs following encounter with self-antigens expressed on thymic medullary epithelial cells. Though controversial, some published evidence suggests regulatory T cells are selected by the same mechanism, in conjunction with signals received through γc-binding cytokines that are also necessary for homeostasis of the cells in the periphery (53-56). Some of the controversy is inherent to the fact that a small percentage of the total Treg population originates from converted conventional T cells in the periphery. A significant amount of published work regarding the role of anti-inflammatory signaling in CD4 T cells through receptors for TGF-β (and STAT3/NFAT downstream) and retinoic acid (RA) suggests conversion takes place primarily in mucosal tissues/areas of exposure to commensal pathobionts (e.g. skin, lungs, and gut) (57-59). The converted cells localize to these areas and produce anti-inflammatory TGF-β and IL-10, contributing to maintenance of barrier homeostasis.

Treg mechanisms of suppression

Much of the research published in the last 10 years in the Treg field has focused on defining mechanisms of suppression, a daunting task given the number and variety of mechanisms proposed. A short list of well defined and intensely studied mechanisms involving either direct cell-to-cell (Treg-to-T_{conv}) or indirect (via APC) suppression by Tregs of both innate and adaptive immunity is outlined in Table 1-1. Work presented in this thesis focuses on three Treg-mediated suppressive mechanisms: IL-10, CTLA-4, and competition with conventional cells for IL-2. A more detailed discussion of these mechanisms will follow at the end of this chapter and in chapters 2 and 4.

Published evidence suggests Tregs exert their suppressive functions both directly and indirectly depending on context (47, 60). There is also evidence to suggest different Treg populations may work concomitantly at different sites or sequentially through different mechanisms – e.g. over the course of a particular infection to limit bystander inflammatory damage inflicted by innate immunity (early) and suppress cellular immunity after infection is cleared (late) (61, 62). Defining a single suppressive mechanism necessary for clearance of a particular infection or tolerance in a particular alloimmune transplant model is therefore unlikely. Suppressive mechanisms likely overlap, but the degree of overlap may be different under different autoimmune or infectious conditions making study of each alone or in combination (coinfection/ heterologous infection/autoimmune manifestation as a result of infection clearance) warranted.

Like conventional T cells, the biology of Tregs is impacted heavily by cell-extrinsic signaling that dictates complex distribution in time and space. This type of complex analysis, used liberally in recent years to study conventional T cell biology, is just beginning to be applied to the study of Tregs. Some extrinsic signals that have garnered attention include responsiveness to chemokines and mutations that effect trafficking ability (63, 64).

Treg-expressed marker	Target	Outcome	Disease models	Salient References
CTLA-4	B7 on DC	Induction of IDO (indoleamine 2,3-deoxygenase) in DC	Tumor, colitis, GVHD (graft versus host disease)	(65-67)
CD39/CD73	ATP/AMP released by damaged DC	Decreased co-stimulation provided by DC	Skin allograft, gastritis, contact hypersensitivity, colitis, multiple sclerosis (MS)	(68, 69)
LAG-3	MHC II on DC	Decreased antigen presentation	NA	(46)
IL-10, IL-35, TGF-β (complexed on cell surface or secreted)	Multiple (Teff, DC, etc.)	Cell cycle arrest / induction of an immunosuppressive phenotype	Tumor, colitis, collagen-induced arthritis	(70-73)
IL-2	Competition w/ Teff	Induction of Teff apoptosis	colitis	(74, 75)
Granzyme-B/ IFN-γ	Teff	Induction of Teff apoptosis	Tumor, GVHD	(76-78)

Table 1-1. Potential Treg mechanisms of suppression. NA = not applicable.

T cell lymphopenia-induced proliferation (LIP) and lymphopenia-driven autoimmunity.

Peripheral T cell deficiency and resultant LIP can occur in a number of physiological and pathological situations. Some of these situations include natural lymphopenic neonatal/prenatal status, involution of the thymus due to aging, iatrogenic

causes such as chemotherapy for cancer and immunotherapy with depleting antibody, and transient or chronic lymphopenia as a result of viral infection (e.g. influenza or HIV) (79, 80). The two general outcomes of T cell LIP are either 1) the loss of TCR diversity, accumulation of autoreactive T cell clones and development of autoimmunity, or 2) homeostasis of cell numbers and suppression of potential autoimmunity by peripheral tolerance mechanisms (81-83).

The concept of T cell reconstitution in response to lymphopenia has been studied extensively using adoptive transfer of naïve T cells into genetically T cell-deficient mice (e.g. mice deficient in recombinase activating gene (RAG), T cell receptor α chain or associated CD3) or mice with induced lymphopenia (irradiation models). Recovery from lymphopenia is characterized by two qualitatively different types of proliferation (84, 85). 'Homeostatic', or slow T cell proliferation, is driven by available resources in the microenvironment, such as the cytokine IL-7 and self-peptide/MHC. 'Spontaneous', or fast T cell LIP, however, is characterized by significant, selective expansion of individual antigen-specific T cell clones with autoaggressive/pathogenic potential, and can be detected in animals free of foreign pathogens (86). Either or both IL-2 and IL-15 may function as stimulators of spontaneous T cell LIP, as mRNA expression of both is up-regulated in mature, TLR-experienced DC, and receptors for which are up-regulated on T cells upon activation (12, 87, 88). T cells that undergo spontaneous LIP, if driven to do so by recognition of microbial antigens (Fig. 1-1), may contribute essential stimulatory cytokines to a subsequent appropriate, foreign pathogen-driven immune response by naïve T cells (89). If peripheral tolerance mechanisms are lost, and if these microbial antigens are

cross-reactive to self, an inappropriate immune response may ensue with autoimmunity as an end result (81, 90).

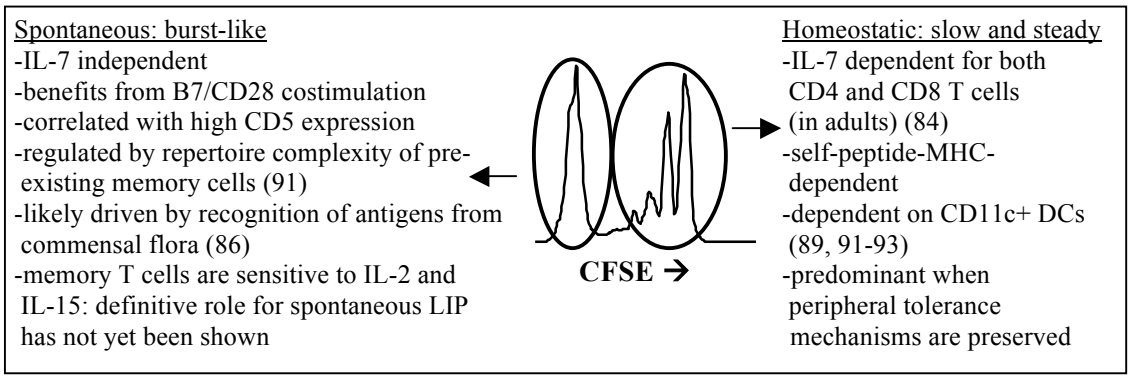


Figure 1-1. 'Homeostatic' and 'Spontaneous' LIP. LIP is typically observed in animals where lymphopenia is caused by gene disruption. Adoptive transfer of naïve CD4 or CD8 T cells into lymphocyte-deficient recipient animals initiates their proliferation, which is most commonly measured by dilution of the dye carboxy-fluorescein diacetate succinimidyl ester (CFSE) (94). The 'homeostatic' form of LIP is characterized by slow and steady proliferation, whereas the 'spontaneous' form is characterized by a rapid burst-like proliferative response that leads to high numbers of cell divisions. These high dividers promptly acquire properties of memory T cells as defined by surface markers, cytokine production potential, and enhanced ability to traffic into peripheral tissues.

Though the validity of genetically lymphopenic mice as physiological models has been called into question, an enormous amount of research on complex aspects of T cell homeostasis has been enabled (95). Autoimmunity involves defects in both branches of the immune response: activation and regulation. The first requirement for development of an autoimmune response is the existence of peripheral T cells with self-reactivity. The second basic requirement is a means for breaking peripheral tolerance. The lymphopenic mouse model is a good one to study autoimmunity in a 'reductionist' way because basic elements fulfilling these requirements can be added individually and events arranged temporally. Several autoimmune models defined in mice that mimic human diseases are characterized by lymphopenia and LIP, including autoimmune gastritis (AIG), thyroiditis, and diabetes (IDDM) (96-99). What may be the most used

and best studied LIP-induced autoimmune mouse model in existence today, however, is the adoptive transfer colitis model pioneered by Fiona Powrie in 1994 (100), in which transfer of $CD4^{+}CD45RB^{high}$ T cells from a normal, healthy wild-type mouse into syngeneic, lymphopenic recipients results in inflammatory bowel disease (IBD) 5-8 weeks later. The important elements of this model – Th1 CD4 T cell responses to enteric flora and dependence of homeostatic cytokines including IL-2 – make it an ideal one for investigation of T cell responses to self-antigens and inflammatory cytokines associated with infection.

Listeria monocytogenes infection model

Listeria monocytogenes (LM) is a gram positive, intracellular bacterial pathogen known to colonize the human gut after ingestion of contaminated food. Individuals who are immunocompromised are especially sensitive to infection with LM, in that both innate and adaptive immunity are important for clearance. In these individuals, LM may quickly spread to other trophic sites, including the spleen, liver, brain, and lymphatics, causing serious illness and death. Trophism in the mouse is very different, in that common lab strains of LM are unable to adhere to the intestinal epithelium (101). Systemic infection in mice mimics that in humans, however, and has demonstrated great utility in assessment of both T cell-dependent and –independent immunity. Injected LM are phagocytosed by macrophages, where they promptly escape the vacuole by secreting the pore-forming haemolysin listeriolysin O (LLO). Once in the cytoplasm, the bacteria replicate and hijack host actin by surface expression of the polymerization

protein ActA, allowing motile bacteria to spread from cell to cell. Escape from the vacuole to the cytoplasm and spread to other APC (namely, DC) exposes LM to the adaptive immune system via presentation of primarily secreted bacterial products to both CD4 and CD8 T cells. An avirulent strain of LM that is deficient in ActA, used liberally for research in recent years, retains its ability to induce memory T cell responses in the mouse (102).

Potential Treg Suppressive Mechanisms

Secretion of immunosuppressive cytokines (e.g. IL-10)

IL-10 plays a part in dampening autoimmune inflammation and in inhibition of antimicrobial immunity. These are related if one considers the best anatomical location for IL-10 detection: the gut (103). IL-10 is crucial to the health of the gut and the best example of this is the transfer colitis model, wherein Asseman and Powrie demonstrated that IL-10 is essential to disease prevention (70). This is a complex immunosuppressive mediator because many cell types, both innate and adaptive, may respond to it (via expression of the four-polypeptide receptor complex), including cells actually making the cytokine. A wealth of published information on the topics of autoimmunity and tumor escape from immunosurveillance suggest Tregs, both thymus-derived and peripherally induced, are a major cellular source of IL-10. Even certain viruses (e.g. EBV) have IL-10 gene homologs and use widespread expression of its receptor to hide from the immune system during the lytic phase (104).

Controversy regarding major cellular sources of IL-10 has been addressed experimentally in recent years with the advent of genetic reporter mouse systems. In 2006, Richard Flavell and colleagues generated and published work on an IL-10 reporter mouse (bred to a Foxp3 knock-in mouse background), which answered many questions regarding Treg-specific IL-10 expression. Specifically, this *tiger* mouse system allowed the Flavell lab to demonstrate conversion of conventional T cells into IL-10-expressing, immuno-regulatory cells in the intestines of lymphopenic hosts upon adoptive transfer (105). In 2007, Casey Weaver and colleagues generated an IL-10/Thy1 T cell reporter mouse to show that Foxp3-negative CD4 T cells also make IL-10 and contribute to dampening gut inflammation (106). Alexander Rudensky complemented the *tiger* model in 2008 by making a Foxp3-conditional IL-10 ablation mouse (107). The ablation mouse model results suggest IL-10 is not required for controlling systemic autoimmunity, but is required for controlling inflammation at sites exposed to environmental antigens (i.e. gut, skin, and lungs).

Chapter 2

Regulatory CD4+CD25+Foxp3+ T cells selectively inhibit the spontaneous form of lymphopenia-induced proliferation of naïve T cells[1]

[1]Reprinted from the Journal of Immunology, Volume 180, pp.7305-7317, 2008, Winstead CJ, Fraser JM, Khoruts A. "Regulatory CD4+CD25+Foxp3+ T cells selectively inhibit the spontaneous form of lymphopenia-induced proliferation of naïve T cells", *Copyright 2008. The American Association of Immunologists, Inc.*

Abstract

Regulatory CD4+CD25+Foxp3+ T cells play a critical role in controlling autoimmunity and T cell homeostasis. However, their role in regulation of lymphopenia-induced proliferation (LIP), a potential mechanism for generation of auto-aggressive T cells, has been poorly defined. Currently, two forms of LIP are recognized: spontaneous and homeostatic. Spontaneous LIP is characterized by fast, burst-like cell cycle activity, and may allow effector T cell differentiation. Homeostatic LIP is characterized by slow and steady cell cycle activity and is not associated with acquisition of an effector phenotype. In this study, we demonstrate that CD4+CD25+Foxp3+ T cells suppress the spontaneous, but not homeostatic, LIP of naïve CD8 and CD4 T cells. However, selective inhibition of spontaneous LIP does not fully explain the tolerogenic role of Tregs in lymphopenia-associated autoimmunity. We show here that suppression of LIP in the lymphoid tissues is independent of Treg-derived IL-10. However, IL-10-deficient Tregs are partially defective in their ability to prevent colitis caused by adoptive transfer of CD4 T cells into RAG-/- mice. We propose that Tregs may inhibit emergence of effector T cells during the inductive phase of the immune response in the secondary lymphoid tissues by IL-10-independent mechanisms. In constrast, Treg-mediated inhibition of established effector T cells does require IL-10. Both Treg functions appear to be important in control of lymphopenia-associated autoimmunity.

Introduction

Peripheral regulation of T cell homeostasis is a critical feature of the adaptive immune system, which ensures adequate T cell population size, TCR diversity, responsiveness to foreign antigens, and self-tolerance. Insults to the immune system that result in transient lymphopenia are common events in the lives of vertebrate organisms, and are typically followed by uneventful immune reconstitution. However, in some clinical settings the drive to re-establish the T cell population size may compromise the diversity of the TCR repertoire and allow emergence of auto-reactive clones, which in turn may lead to immunodeficiency and autoimmunity (81, 108-110). Thus, understanding the mechanisms that regulate T cell homeostasis has important clinical applications.

Experimentally, lymphopenia-induced proliferation (LIP) of naïve T cells is commonly studied using adoptive transfer systems with T cell-deficient recipients. Recently, two forms of LIP have been defined: homeostatic and spontaneous (84). Homeostatic LIP is slow and dependent on IL-7. In contrast, spontaneous LIP is rapid and independent of IL-7. Self-peptide/MHC ligand stimulation benefits both forms of LIP. However, the two forms of LIP may differ in the strength of TCR stimulation experience. In general, T cells with higher avidity for self-ligands undergo more vigorous LIP (111-113). Thus, it is possible that the experience of T cells undergoing spontaneous LIP may be comparable to full cognate antigenic stimulation, which benefits from CD28 co-stimulation (79, 86, 89, 114, 115). In fact, the highly divided T cells emerging from LIP preferentially acquire ability to produce effector cytokines and

migrate into peripheral tissues (79, 84, 86, 89, 114, 115). This phenotype, combined with likely higher degree of self-reactivity among the high dividers, suggests that the majority of auto-reactive pathogenic T cell clones are generated by spontaneous proliferation during LIP.

While competition for positive resources such as cytokines and self-ligands in T cell homeostasis has been studied in some detail, much less is known about potential inhibitory factors. Regulatory CD4+CD25+Foxp3+ T cells (Tregs) have long been suspected to play a role in T cell homeostasis, and have been demonstrated to be suppressive in multiple animal models of autoimmunity that involve lymphopenic hosts. However, their role in controlling LIP has been unclear and controversial (116). Initial studies failed to demonstrate an effect of co-transfer of Tregs on the LIP of CFSE-labeled CD4+CD25- T cells as measured by dilution of the dye (117). Other studies noted that co-transfer of Tregs markedly decreased the homeostatic plateau population size of CD4+CD25- T cells, but allowed at least some LIP (118, 119). Finally, recent studies demonstrated that Tregs inhibit proliferation, survival and differentiation of CD4+CD25- responder T cells following adoptive transfer into lymphopenic hosts (114, 120).

Failure to distinguish between homeostatic and spontaneous forms of LIP is one potential reason for conflicting reports on the suppressive potential of Tregs. In this study, we report that Tregs can selectively inhibit the spontaneous form of LIP, but have little effect on the homeostatic form. This suppressive ability cannot be attributed to mere competition for TCR signals, because CD4 Tregs inhibit spontaneous LIP of CD8 T cells, which receive TCR signals from MHC class II and MHC class I molecules,

respectively. Previously, IL-10-deficient Tregs have been reported to be unable to inhibit expansion of CD4 T cells in RAG-/- hosts (118). Therefore, we tested whether Treg-derived IL-10 could mediate inhibition of LIP. We found that IL-10-deficient and wild-type Tregs are indistinguishable in their ability to suppress LIP within secondary lymphoid tissues. Interestingly, IL-10-deficient Tregs were only partially effective in preventing colitis caused by CD4 T cell responders. Incomplete suppressive ability of IL-10-deficient Tregs is consistent with conflicting earlier reports on requirements for Treg-derived IL-10 control of colitis (119, 121). Our results suggest a two-step model for the role of Tregs in lymphopenia-associated autoimmunity. First, by selective inhibition of spontaneous LIP, Tregs limit emergence of pathogenic T cells. Second, Tregs act within sites of inflammation to suppress activity of pathogenic T cells that do escape into the periphery. The two suppressive functions appear to be mediated by different mechanisms, and both may be required for optimal control of autoimmunity associated with lymphopenia.

Materials and Methods

Mice

C57BL6 (B6) and CD45.1 congenic mice were purchased from the National Cancer Institute (Frederick, MD). Recombinase-deficient (RAG-1$^{-/-}$; hereafter referred to as RAG$^{-/-}$), Thy1.1 congenic, and IL-10$^{-/-}$ mice on the B6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). RAG-1$^{-/-}$ OT-I TCR-transgenic mice (Tg)

(122) were bred onto CD45.1 and Thy1.1 congenic backgrounds. All mice used were generally 6-12 weeks of age. All animals were maintained in a specific pathogen-free facility in microisolator cages with filtered air according to the National Institutes of Health guidelines. All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Adoptive transfer and cell preparations

Unless otherwise specified, donor T cells were collected from secondary lymphoid tissues (axillary, brachial, cervical, mesenteric, and inguinal lymph nodes, and spleen). $CD44^{low}$ CD8 and control $CD44^{low}$ CD4 T cells were purified in two stages: first, CD8 or CD4 T cells were prepared by negative selection against CD8 or CD4, MHC class II, CD11b, and B220 (all Abs labeled with FITC) using anti-FITC BioMag particles (Polysciences, Warrington, PA), and in some cases also anti-CD25-, glucocorticoid-induced TNFR (GITR), and CD103-FITC, followed by depletion of $CD44^{high}$ cells using magnetic microbeads (Miltenyi Biotec, Auburn, CA), as previously described (123). Briefly, the purified CD8 or CD4 T cells were suspended in labeling buffer (2% FCS in PBS), incubated with 0.004 μg anti-CD44-FITC (eBioscience, San Diego, CA) per 10^6 cells for 15 minutes, washed, and labeled with anti-FITC magnetic microbeads. The negative fraction was collected following Miltenyi Biotec magnetic column separation. $CD25^+$ T cells were prepared using positive selection with anti-CD25 biotinylated mAb, PC61, and streptavidin-labeled magnetic microbeads (Miltenyi Biotec). $RAG^{-/-}$ OT-I lymph nodes and spleens or lymph nodes alone were harvested, and purified CD8 T cells were labeled with CFSE and i.v. injected into host

mice. Labeling with CFSE was done using a technique described previously (94).

FACS analysis

Mice were sacrificed and perfused with PBS before removal of lymph nodes, spleen, liver, lungs, and colons. All secondary lymphoid tissues were disrupted by mashing with a syringe. Livers and lungs were digested in collagenase D (Roche Applied Science), 400 U/ml^{-}1 for 30 min at 37°C. In some cases, the collagenase medium was supplemented with 10 μg/ml^{-}1 brefeldin A (Sigma-Aldrich) to prevent lymphokine secretion. Liver cells were separated using a 40/100 Percoll gradient, and the hematopoietic cells were collected from the interface. Colons were washed with PBS, cut into small segments, and digested before staining for FACS. Intracellular staining for cytokines was done following stimulation with PMA/ionomycin and fixation. Specific T cell subsets were identified using fluorochrome-labeled Abs against CD4, CD8, and congenic markers such as anti-Thy1.1/Thy1.2 and anti-CD45.1/CD45.2. All anti-cytokine Abs (IL-2, IL-17A, IFN-γ, and isotype controls) were purchased from eBioscience. Granzyme B was stained intracellularly using anti-human granzyme B Abs (Caltag Laboratories). Staining for Foxp3 was done using an eBioscience kit and instructions provided by the manufacturer. Absolute numbers of T cells within various tissues were calculated using PKH reference beads (Sigma-Aldrich). Relative numbers of CD4 T cells of specific subtype were calculated in comparison to the number of total CD4 T cells in recipient groups that did not receive Tregs.

Induction and assessment of colitis

Colitis was induced by i.v. adoptive transfer of 1 x 10^5 $CD4^+CD25^-CD103^-$ $CD44^{low}GITR^{low}$ cells purified using magnetic microbeads from wild-type donor mice, as described above, into $RAG^{-/-}$ animals. For some of the recipient mice, 0.5-2 x 10^6 $CD4^+CD25^+$ Tregs were transferred 1 wk before the transfer of naïve CD4 T cells, unless indicated otherwise. The animals were monitored clinically by weights and signs of colitis and systemic toxicity. After sacrifice, a 1 cm segment of the distal-most part of the colon was removed and fixed in 10% buffered formalin. Parrafin-embedded sections were cut and stained with H & E. Severity of colitis was scored in a blinded fashion according to a scale similar to that previously described (121): grade 0, normal histologic appearance; grade 1, minimal scattered inflammatory cell infiltrates within the lamina propria, with or without minimal epithelial hyperplasia; grade 2, mild to moderate, scattered inflammatory infiltrates, sometimes extending into the submucosa, with mild to moderate epithelial hyperplasia and and mild to moderate depletion of mucin from goblet cells; grade 3, moderate inflammation in the lamina propria, sometimes transmural, occasional crypt abcesses, moderate to severe epithelial hyperplasia and mucin depletion; grade 4, severe inflammatory infiltration of lamina propria, transmural inflammation, abundant crypt abcesses, occasional ulceration, marked epithelial hyperplasia, and mucin depletion; grade 5, severe inflammation with diffuse loss of epithelium.

Results

Tregs inhibit spontaneous LIP of naïve CD8 T cells

Adoptive transfer of naïve CD4 or CD8 T cells into lymphocyte-deficient recipient animals initiates their proliferation. The use of CFSE-labeled T cells in adoptive transfer protocols allows their proliferation to be easily measured by dilution of the CFSE dye (94). Two forms of LIP are currently recognized and are typically observed in animals where lymphopenia is caused by genetic disruption of T cell development: 'homeostatic' and 'spontaneous' (84). The rapid, burst-like proliferation characteristic of spontaneous LIP leads to loss of detectable CFSE in responder T cells ($CFSE^-$). In contrast, T cells undergoing homeostatic LIP, which is slow and steady, have detectable CFSE ($CFSE^+$). To demonstrate these two forms of LIP, we measured CFSE content subsequent to adoptive transfer of CFSE-labeled naive CD8 T cells into lymphocyte-deficient $RAG^{-/-}$ mice. We used two different responder CD8 T cell populations: naive polyclonal T cells and monoclonal OT-I TCR Tg $RAG^{-/-}$ T cells (OT-I T cells). The two distinct forms of LIP were exhibited by both responder populations. Ten days after transfer, two-thirds of polyclonal CD8 responder T cells had lost detectable CFSE signal, indicating that they were undergoing spontaneous LIP (Fig. 1*A*, *left panel*). A significant fraction of OT-I T cells had also lost detectable CFSE signal 8 days following adoptive transfer into $RAG^{-/-}$ mice, although the majority of these responders retained measurable CFSE content characteristic of homeostatic LIP (Fig. 1*A*, *right panel*).

T cells undergoing spontaneous LIP have been shown to promptly acquire

properties of memory T cells as defined by surface markers, cytokine production potential, and enhanced ability to traffic into peripheral tissues (79, 115, 124-126). It is likely that this fraction of T cells experiences activation similar to that of cognate Ag stimulation, and is enriched for autoreactive specificities. Therefore, we wished to test whether Tregs have different effects on the two different forms of LIP. We focused the initial studies on CD8 responder T cells to avoid effects of Tregs that could be attributed to competition for self-ligand stimulation.

To measure LIP, we transferred varying numbers of CFSE-labeled polyclonal naive CD8 T cells or monoclonal OT-I T cells into $RAG^{-/-}$ recipients. Eight or 10 days later, we measured the CFSE dye content of adoptively transferred T cells in the secondary lymphoid tissues. Interestingly, regardless of the number of transferred polyclonal CD8 T cells, we always observed a similar percentage of $CFSE^{-}$ T cell responders. (Fig. 1*B*, *left panel*). However, in the case of OT-I T cells, the emergence of the $CFSE^{-}$ fraction was more sensitive to the input number, and it could not be detected if we transferred $>5 \times 10^6$ T cells per recipient (Fig. 1*B*, *right panel*). The high input of OT-I T cells only allowed homeostatic LIP to take place. This observation is consistent with the idea that spontaneous LIP requires strong TCR stimulation, which can become limiting for large numbers of monoclonal T cells competing for the same peptide/MHC signals.

Next, we tested the effects of Treg cells on LIP by first transferring Treg cells into $RAG^{-/-}$ recipients, then 1 wk later transferring CFSE-labeled naive CD8 T cell responders into the same recipients, $RAG^{-/-}$ mice that had not received a pretransfer of Treg cells, or wild-type mice. Presence of Tregs greatly decreased the proportion of

$CFSE^-$ T cells arising from the polyclonal input CD8 T cell population or OT-I cells transferred in relatively low numbers (Fig. 2, *A*, *left* and *middle panels*, and *B*, *top left* and *middle panels*). In contrast, Tregs had little effect on the CFSE content of T cells undergoing the slower homeostatic form of LIP (Fig. 2*B*, *lower left* and *middle panels*). Furthermore, Tregs did not inhibit LIP of OT-I T cells transferred at high input number (Fig. 2, *A*, *right panel*, and *B*, *bottom right panel*), a condition that only allows the homeostatic form of LIP. These results indicated that Tregs selectively inhibited emergence of the $CFSE^-$ fraction, but had no effect on the profile of the $CFSE^+$ responders.

In addition, we tested the effects of Tregs on some functional characteristics of responder CD8 T cells undergoing LIP. This included expression of granzyme B and homing into peripheral tissues. We first transferred Treg cells into $RAG^{-/-}$ recipients, then one week later transferred CFSE-labeled naive polyclonal CD8 T cell responders into the same recipients or into $RAG^{-/-}$ mice that had not received a pretransfer of Treg cells. In the absence of Tregs, the $CFSE^-$ fraction formed the majority in the secondary lymphoid tissues and was overwhelmingly dominant in nonlymphoid tissues such as liver and lung (Fig. 3*A*, *top panels*). In the presence of Tregs the fraction of $CFSE^-$ T cells significantly diminished (Fig. 3, *A*, *bottom panels*, and *B*, *top panels*), and the total numbers of $CFSE^-$ T cells decreased more than an order of magnitude in both lymphoid and nonlymphoid tissues (Fig. 3*C*, *left panel*). In contrast, the numbers of $CFSE^+$ T cells were not altered by the presence of Tregs (Fig. 3*C*, *right panel*). Furthermore, the presence of Tregs did not alter the CFSE content of $CFSE^+$ T cells in the lymphoid tissues, although it did seem to increase the CFSE content of $CFSE^+$ T cells slightly in

the nonlymphoid tissues (Fig. 3*B*, *bottom panel*). Notably, only the $CFSE^-$ fraction of polyclonal CD8 T cells expressed significant levels of granzyme B (Fig. 3*A*), suggestive of their more differentiated, effector phenotype (127). Interestingly, both low and high dividers found in the liver in the presence of Tregs expressed granzyme B, which was not the case for other peripheral tissue sites, such as lungs (Fig. 3*A*, *rightmost lower panel*). This may relate to the observation of liver being a site of accumulation for effector T cells destined to undergo apoptosis (128).

Naïve CD4+CD25- T cells enhance spontaneous LIP of naïve CD8 T cells

We considered that the observed inhibition of spontaneous LIP by Tregs may be due to consumption of some limiting resources, such as cytokines, that are common to all T cells. Thus, we next tested the effects of conventional CD4 T cells on LIP of naive CD8 T cells by first transferring naive $CD4^+CD25^-$ T cells into $RAG^{-/-}$ recipients, then 1 wk later transferring CFSE-labeled naive polyclonal CD8 or OT-1 responders into the same recipients or into $RAG^{-/-}$ mice that had not received a pre-transfer of CD4 cells. The conventional CD4 T cells rapidly expanded during the week before the transfer of CD8 T cells to match or exceed the numbers of cells we observed following Treg transfers (data not shown). Notably, the mice did not display any clinical signs of autoimmunity, such as colitis, that may be seen over a longer time course (129). In contrast to the effects we observed with Tregs, we found that the conventional CD4 T cells actually enhanced spontaneous proliferation of CD8 T cells. In fact, on day 10 the $CFSE^-$ fraction dwarfed the $CFSE^+$ population in mice that received conventional CD4 T cells (Fig. 4*A*), and the total numbers of CD8 T cells were considerably greater in these

animals compared with animals that did not receive a pretransfer of conventional CD4 T cells (Fig. 4*B*, *center panel*). The results were similar for polyclonal and OT-1 CD8 T cell responders. Furthermore, CD8 T cells that underwent spontaneous LIP in the presence of conventional CD4 T cells also benefited in terms of differentiation, as measured by greater expression of granzyme B (Fig. 4*B*, *right panel*). This differentiation was evident even for highly divided OT-1 cells, which express only minimal levels of granzyme B in absence of CD4 help (Fig. 4*B*, *right panel*). Interestingly, the CFSE content of the $CFSE^{+}$ CD8 T cells was greater in the presence of conventional CD4 T cells (Fig. 4, *A* and *B*, *left panel*), suggesting that the two T cell populations do compete for common resources that are important for the homeostatic form of LIP.

IL-10 production by Tregs is not required to inhibit LIP of CD8 T cells

Next, we wished to probe the mechanism of Treg-mediated inhibition of spontaneous LIP. Although multiple mechanisms for suppressive function of Tregs have been proposed to operate in different systems, these mechanisms remain very poorly understood. We decided that IL-10 production by Tregs is one important mechanism to explore, because it has been reported that IL-10-deficient Tregs cannot inhibit expansion of CD4 T cells following adoptive transfer into RAG-/- hosts (118). Thus, we tested whether IL-10-deficient Tregs could inhibit LIP of naïve CD8 T cells. Because IL-10-deficient mice develop inflammatory bowel disease over time, we exclusively used relatively young mice at 6 weeks of age or less as donors of Tregs. Analyses of Tregs from the lymph nodes and spleens of IL-10-deficient mice and wild-

type mice indicated that the purity of Tregs isolated from both donors and the levels of Foxp3 expression in both types of mice were comparable (Fig. 5*A*). In addition, wild-type and IL-10-deficient Tregs expressed equivalent levels of CTLA-4, GITR, and CD69 (Fig. 5*B*), and had similar ability to undergo their own homeostatic proliferation as evidence by comparable CFSE profiles (Fig. 5*C*, *left panel*), and numbers of cells recovered following expansion (Fig. 5*C*, *right panel*).

After confirming that Tregs from IL-10-deficient mice were phenotypically similar to those from wild-type mice, we examined the effects of IL-$10^{-/-}$ Tregs on LIP of naive polyclonal CD8 T cells in $RAG^{-/-}$ mice. We first transferred wild-type or IL-$10^{-/-}$ Treg cells into $RAG^{-/-}$ recipients, then 1 wk later transferred CFSE-labeled naive CD8 T cell responders into the same recipients or into $RAG^{-/-}$ mice that had not received a pretransfer of Treg cells. We found that wild-type and IL-10-deficient Tregs were comparable in their ability to suppress spontaneous LIP of CD8 T cells, as evidenced by similarly decreased fraction of $CFSE^{-}$ responders in presence of either type of Tregs (Fig. 6*A*). The suppression resulting in decreased total numbers of CD8 T cells in the presence of either wild-type or IL-10-deficient Tregs in the secondary lymphoid tissues was even more dramatic in the periphery, where in absence of Tregs the overwhelming majority of responders belonged to the $CFSE^{-}$ fraction (Fig. 6*B*). The experiments suggest that inhibition of spontaneous LIP by Tregs within the lymphoid tissues limits emergence of effector T cells capable of trafficking into the periphery.

IL-10 production by Tregs is not required to inhibit LIP of CD4 T cells

We considered that the lack of any defect in suppressive effects of IL-10-deficient Tregs on LIP of CD8 T cells might be due to a fundamental difference between CD8 and CD4 responders. This includes their relative pathogenicity. Adoptive transfer of naive polyclonal CD4 T cells can lead to fatal autoimmunity or immunopathology, which includes colitis and pneumonitis (129, 130). In contrast, we observed $RAG^{-/-}$ recipients of naive polyclonal CD8 T cells for 3 mo and did not note any disease (data not shown). Thus, we tested the ability of Tregs to inhibit LIP of polyclonal CD4 T cells using the identical experimental design we used for CD8 responders above. We first transferred wild-type or $IL\text{-}10^{-/-}$ Treg cells into $RAG^{-/-}$ recipients, then 1 wk later transferred CFSE-labeled naive CD4 T cell responders into the same recipients or into $RAG^{-/-}$ mice that had not received a pretransfer of Treg cells. CFSE content was measured 7 days after the adoptive transfer of CD4 responders (Fig. 7*A*). Presence of either IL-10-deficient or wild-type Tregs resulted in a decreased proportion of $CFSE^{-}$ CD8 T cell responders (Fig. 7*B*, *upper panels*). Interestingly, there was a trend toward greater CFSE content in the $CFSE^{+}$ fraction in the presence of Tregs, at least in the mesenteric lymph nodes (Fig. 7*B*, *lower right panel*). This suggests that there may be some competition for common resources among Tregs and conventional CD4 T cells. However, the most marked effect of Tregs, either IL-10-deficient or wild type, was on the numbers of $CFSE^{-}$ responders, which were significantly decreased in the presence of both types of Tregs (Fig. 7*C*). These data indicated that Tregs selectively inhibited the spontaneous form of LIP of naive CD4 cells, and that wild-type and IL-10-deficient Tregs were no different in their suppressive potency.

Treg-derived IL-10 plays an important role in control of colitis induced by CD4 T cells in RAG-/- mice

Although our data demonstrated that Treg-derived IL-10 is not involved in control of LIP within lymphoid tissues in the absence of disease, we questioned whether it may become important after onset of inflammatory disease, which itself may perpetuate expansion of CD4 T cells if left uncontrolled. To test this possibility, we first transferred wild-type or IL-10$^{-/-}$ Treg cells into RAG$^{-/-}$ recipients, then 1 wk later transferred naive CD4 T cell responders into the same recipients or into RAG$^{-/-}$ mice that had not received a pretransfer of Treg cells. We then assessed the severity of colitis following adoptive transfer. RAG$^{-/-}$ mice that received no Tregs developed severe colitis and had to be sacrificed at 6–9 wk after transfer of conventional CD4 T cells, according to preset criteria. RAG$^{-/-}$ mice that received IL-10-deficient Tregs before the transfer of CD4 T cells developed colitis of intermediate severity, while RAG$^{-/-}$ mice that received wild-type Tregs before the transfer of CD4 T cells developed no disease (Fig. 8). Significantly different weights were noted among the groups, expressed as percent of initial weight (Fig. 8*A*, *left panel*), and they correlated well with the colitis histology scores (Fig. 8, *A*, *right panel*, and *B*).

In addition to measuring the effects of wild-type and IL-10-deficient Tregs on clinical colitis caused by CD4 T cells in RAG$^{-/-}$ mice, we analyzed the effector cytokine profiles generated by CD4 T cells in the same animals. T cells taken from mesenteric lymph nodes or colons were stimulated with PMA/ionomycin in vitro, and cytokine production was measured by intracellular staining. Most cells produced either IFN- or

IL-17, although some cells produced both cytokines simultaneously (Fig. 9, *A* and black bars in *B*). Tregs, regardless of their ability to produce IL-10, markedly inhibited expansion of IFN--producing CD4 T cells. This was evidenced by smaller fractions and total numbers of IFN--producing CD4 T cells in the mesenteric lymph nodes (Fig. 9*B*, *top left panels*). However, IL-10-deficient Tregs were less effective than wild-type Tregs in suppressing infiltration of colons by IFN--producing CD4 T cells (Fig. 9*B*, *bottom left columns)*. Interestingly, both types of Tregs were only modestly effective in reducing the numbers of Th17 cells compared with Th1 cells. In fact, the presence of wild-type or IL-10-deficient Tregs inverted the IFN--producer/IL-17-producer ratio in favor of Th17 T cells. Nevertheless, similarly to the effect seen on IFN--producing CD4 T cells, wild-type Tregs effectively prevented infiltration of colons by IL-17-producing CD4 T cells, while IL-10-deficient Tregs failed to do so. These findings, combined with no demonstrable difference in the ability of wild-type and IL-10-deficient Tregs to control LIP within lymphoid tissues, suggest that Treg-derived IL-10 is important primarily in control of established pathogenic effectors in the peripheral tissues.

It is important to note that suppressive function of Tregs in experimental colitis experiments is typically measured following a cotransfer with 10^5 conventional CD4 T cells in a 1:1 or even 1:10 Treg-responder ratio. This contrasts with our staggered protocol using more Treg cells. However, we had determined early in our work that cotransfer of Tregs in relatively small numbers does not inhibit LIP (data not shown). We did the above colitis experiments specifically trying to tease out the potential contribution of Treg-mediated inhibition of spontaneous LIP in pathogenesis of experimental disease. However, we did attempt to compare our results reported above

(Fig. 8) with those that would be obtained using a cotransfer protocol. We ran pretransfer and cotransfer Treg/responder T cell adoptive transfer protocols in parallel. In one set of mice, we first transferred 1 x 10^6 wild-type or IL-$10^{-/-}$ Treg cells into $RAG^{-/-}$ recipients (on day –7), then 1 wk later transferred naive CD4 T cell responders into the same recipients (on day 0). A second set of $RAG^{-/-}$ mice that had not received a pretransfer of Treg cells received a cotransfer of 5 x 10^5 or 1 x 10^5 wild-type or IL-$10^{-/-}$ Treg cells and 1 x 10^5 naive CD4 T cell responders on day 0. A control set of $RAG^{-/-}$ mice received only naive CD4 T cell responders on day 0. When assayed simply by weight, the mice benefited from both wild-type and IL-10-deficient Tregs received through both the pretransfer or the cotransfer protocols (Fig. 10*A*, *left panel*). However, in both adoptive transfer protocols, IL-10-deficient Tregs failed to prevent significant histological colitis activity, while wild-type Tregs completely abolished histologic colitis (Fig. 10*A*, *right panel*). As found above, these data indicate that IL-10-deficient Tregs at all doses tested were less successful in prevention of disease than wild-type Tregs. Higher histology scores correlated with higher total numbers of responder T cells isolated from the colon (Fig. 10*B*, *left panel*), as well as with higher numbers of T cells capable of producing IFN- and/or IL-17 (data not shown).

An interesting caveat of these titration colitis experiments, which were performed in the later phase of this project, was that a longer period was required for colitis induction than in earlier studies. In our early experiments shown in previous figures, diarrhea was evident by 3–4 wk after transfer of responder T cells. Interestingly, we saw that the ability of Tregs to prevent colitis in early experiments was relatively inefficient, when simultaneously cotransferred with responders in 1:1 ratio (data not

shown). However, in these later experiments (Fig. 10), the colitis induction period was 9–10 wk. This longer induction period was consistently observed by several individual workers in the laboratory in various individual experiments, and may reflect a change in enteric flora in our animal facility. During this long induction period, Tregs likely have sufficient time to expand even from low input numbers to occupy their homeostatic niche, which is reflected by similar numbers of Tregs found in the lymphoid tissues at the end of the experiment (Fig. 10*B*, *right panel*).

Discussion

Although regulatory CD4+CD25+ T cells were originally discovered in the context of autoimmunity associated with lymphopenia (131-133), surprisingly they were also initially reported to have no inhibitory effects on T cell LIP (116, 117). At least two distinct forms of LIP have been recognized since: spontaneous and homeostatic (84). These are readily distinguished in short-term experiments by the content of CFSE dye subsequent to the adoptive transfer of CFSE-labeled T cells. The spontaneous form is characterized by rapid, burst-like T cell proliferation that results in complete loss of CFSE. It is also associated with acquisition of potentially pathogenic effector phenotypes. In contrast, the homeostatic form is characterized by a slow and steady rate of cell division and does not usually lead to acquisition of effector functions. We show here that Tregs selectively inhibit the spontaneous form of LIP, but spare the homeostatic form of LIP. This finding does resolve some of the conflict in the literature on the potential role of Tregs in controlling LIP. First, the spontaneous form of LIP can

be easily missed if CFSE is used as the sole marker of adoptively transferred cells. Second, not all models of lymphopenia support spontaneous LIP. Although spontaneous LIP is readily seen following adoptive transfer of T cells into animals with a complete congenital deficiency of T cells, it is largely absent in irradiation-induced lymphopenia (86, 89, 134). In fact, our results provide one explanation for the latter finding: residual endogenous Tregs may be sufficient to block spontaneous LIP following irradiation.

After our original observation that Tregs can inhibit LIP of CD4 T cells (114), we considered that Tregs may simply compete for TCR signals with the CD4 responders. Indeed, Treg TCRs are generally thought to have higher affinities for self-peptide/MHC complexes compared with TCRs of conventional CD4 T cells (135). To test the hypothesis of competition for peptide/MHC signals, we focused our initial studies here on CD8 T cells as responders, because it is unlikely that there is substantial overlap between peptide/MHC signals seen by CD8 T cells and CD4 Tregs. Indeed, a different mechanism is likely responsible for the ability of Tregs to inhibit the spontaneous form of LIP experienced by CD8 T cells. However, competition for peptide/MHC signals may be one reason for the very modest inhibition of homeostatic LIP experienced by naïve CD4 T cells (Fig. 2-7B). It might also explain complete Treg-mediated inhibition of LIP by several monoclonal CD4 T cell populations reported previously (114, 120). However, our results here show that Tregs inhibit the spontaneous form of LIP experienced by polyclonal CD4 T cells more than the homeostatic form, and it is reasonable to speculate that this inhibition is mediated by a mechanism common for both CD4 and CD8 responders.

Molecular mechanisms of Treg-mediated suppression are still poorly understood. It is likely that multiple mechanisms operate simultaneously, and those that are critical *in vitro* may play only secondary roles *in vivo*. For example, Tregs were described to suppress IL-2 production and expression of CD25 by responder T cells during *in vitro* culture by a mechanism that required direct cell-cell contact (136). However, direct *in vivo* imaging studies failed to detect physical interactions between Tregs and responder T cells. Instead, Tregs were seen to limit stable conjugation between responder T cells and dendritic cells (DCs) (137, 138). Interestingly, these effects do not interfere with early activation events in the responder T cells, including production of IL-2 (139, 140). Some of the inhibitory mechanisms mediated by Tregs that have been shown to play important roles *in vivo* include production of immunosuppressive cytokines such as IL-10 (141, 142) and TGF-β (73, 143), induction of indoleamine 2,3-dioxygenase (IDO) via CTLA-4:B7 engagement in certain DC subsets (144, 145), and generation of anti-proliferative molecules such as adenosine (69, 146, 147).

We decided to focus our initial exploration of Treg-mediated suppression of LIP on IL-10 because it has been reported previously that IL-10-deficient Tregs fail to inhibit expansion of CD4 T cells in RAG-/- hosts (118). However, we found no defect in the ability of IL-10-deficient Tregs to control LIP of CD8 or CD4 T cells measured within the secondary lymphoid tissues. Interestingly, IL-10-deficient Tregs were significantly impaired in their ability to control colitis. This result can be understood if we recognize that Tregs function differently in different anatomic locations. We propose the following model (Fig. 2-11). During the inductive phase of the immune

response, which takes place within the secondary lymphoid tissues, Tregs limit oligoclonal expansion of T cells experiencing cognate antigen stimulation. This function is IL-10-independent; in fact, Tregs localized within secondary lymphoid tissues produce little IL-10 (139, 148). However, the suppressive effects of Tregs in the lymphoid tissues are incomplete, and some effector T cells are generated even in their presence. These effectors leave the lymphoid tissues and migrate into the periphery. Once there, established effectors can become activated again and trigger inflammation. At this phase of the immune response Tregs suppress the activity of established pathogenic effectors by mechanisms that at least partially depend on their ability to produce IL-10. This model fits with the observation that Tregs produce IL-10 within inflamed tissues, but not within secondary lymphoid tissues (139, 148). This model predicts that wild-type Tregs limit emergence of pathogenic T cells from the lymphoid tissues and inhibit their activity in the periphery. Therefore, no colitis is observed in their presence. However, IL-10-deficient Tregs are only partially effective. Although they limit emergence of pathogenic T cell in the lymphoid tissues, they fail to suppress pathogenic activity of effector T cells in the periphery. The model explains the intermediate severity of colitis observed in our experiments with IL-10-deficient Tregs (Figs. 2-8 through 2-10). The model is also consistent with failure to rescue scurfy mice (*Foxp3*^{sf}) from fatal autoimmunity by adoptive transfer of Tregs after the immediate neonatal period. Adoptive transfer of wild-type Tregs into neonatal *Foxp3*^{sf} mice fully protects them from disease (49, 149, 150). However, adoptive transfer of Tregs into *Foxp3*^{sf} mice on day 3 of life or later merely delays their eventual death from autoimmune disease (151). We suspect that in the first case, Tregs limit emergence of

pathogenic T cells and further suppress activity of the few that do escape into the peripheral tissues. However, in the second case, the large number of differentiated pathogenic effectors cannot be completely controlled in the periphery.

It is important to note that our experimental system differs somewhat from the usual protocols for adoptive transfer with Tregs for disease suppression. Typically, Tregs are co-transferred simultaneously along with the responder T cells into the RAG-/- recipients. In contrast, in most experiments here we transferred 0.5-2 x 10^6 Tregs one week before the responder T cells were transferred. Tregs occupy a homeostatic compartment separate from other T cells (119, 152-154) and during this week they expand to reach near plateau numbers (data not shown). Our staggered adoptive transfer protocol is similar to one used by Shen et al. who were also able to demonstrate control of LIP by Tregs (120). The relatively large number of polyclonal Tregs is important for revealing their ability to suppress LIP. In contrast, co-injection of a relatively small number of Tregs fails to control the initial proliferation and expansion of CD4 responders (155). Therefore, it is likely that in co-transfer experiments, Tregs exert most of their suppressive effects directly in the tissues. This may explain the ability of Tregs to cure established CD4 T cell-induced colitis in RAG-/- mice, and the failure of IL-10-deficient Tregs to treat this disease (148).

It is reasonable to ask whether inhibition of LIP by Tregs plays a significant role in inhibition of experimental colitis and other types of autoimmunity. We did see a trend toward less efficient disease suppression when the lowest numbers of Tregs were used, as evidenced by greater numbers of responder T cells in the colon (Fig. 10*B*) and greater numbers of polarized Th1 and Th17 effectors (data not shown). Nevertheless, in their

totality our results can also be interpreted to suggest that inhibition of LIP within lymphoid tissues is not an essential mechanism of disease suppression. Instead, suppression of established effector T cells may be sufficient. However, this would contradict recent demonstration of poor suppression of colitis by CCR7-deficient Tregs, which fail to migrate into lymph nodes and fail to inhibit generation and expansion of colitogenic T cell clones (64). These data do support our interpretation: Tregs do play an important role during the inductive phase of the immune response within the lymphoid tissues, and they have to be present in large numbers to be effective during this stage. This condition was likely unmet in simultaneous cotransfer experiments where there was insufficient time for Treg expansion before colitis induction.

It should be noted that clinically important lymphopenic states with relative sparing of the Treg compartment are common. We have already mentioned that irradiation-induced lymphopenia, common in cancer clinics, is rarely associated with autoimmunity. Similarly, Tregs may be spared in lymphopenia caused by viral infections, which results in impaired viral clearing (156). Increased numbers of Tregs have been observed in patients with HIV infection (157, 158). In this case, we speculate that residual Tregs may protect patients being treated by highly active anti-retroviral therapy against the immune reconstitution syndrome.

Finally, it is interesting to consider that selective inhibition of spontaneous LIP might also benefit the overall TCR diversity achievable during recovery from a lymphopenic state by LIP. Currently, it is not known whether responders undergoing spontaneous and homeostatic forms of LIP compete for any overlapping resources. If they do, then it is possible for a relatively small number of T cells prone to spontaneous

LIP to dominate the T cell repertoire following recovery from a lymphopenic state. For example, although T cells undergoing spontaneous LIP do not require IL-7, they still express similar levels of the IL-7Rα as T cells undergoing homeostatic LIP, and theoretically might steal IL-7 away from them (84). It is also possible that T cell undergoing spontaneous LIP may compete for TCR signals. In fact, it has been shown that at least some TCR Tg T cells with relatively high avidity for self-ligands can inhibit homeostatic proliferation of other TCR Tg T cells with lower avidity for self-ligands following co-transfer into lymphopenic hosts (112). Furthermore, memory T cells generated by immunization with antigen or LIP in RAG-/- recipients can inhibit LIP of naïve responders if the two populations have an overlap in TCR specificity (91, 159). Therefore, it is possible that the expansion of T cells by spontaneous proliferation can occur at the expense of homeostatic proliferation. In this case, the spontaneous form of LIP may be detrimental not only because it is more likely to give rise to pathogenic T cells, but also because it may limit immune fitness by constraining the homeostatic form of LIP. Future studies are needed to test this hypothesis.

FIGURE 2-1. Naive polyclonal and monoclonal transgenic OT-1 CD8 T cells undergo homeostatic and spontaneous forms of LIP. OT-1 $RAG^{-/-}$ or $CD44^{low}$CD8 T cells were CFSE-labeled and injected in varying numbers into $RAG^{-/-}$ recipients on day 0. Lymph nodes were harvested and analyzed by FACS on day 8 (OT-1 cells) or 10 (polyclonal cells). CD8 responders were gated using CD45.1 or Thy1.1 congenic markers and CD8. *A*, Representative CFSE profiles of CD8 responder T cells in the lymph nodes from individual animals following adoptive transfer of 1 x 10^6 cells. The arrows within histograms indicate the control location of the signal generated by control, undivided T cells 1 day after transfer. *B*, Data shown are the mean percentage of responder T cells that have lost detectable CFSE signal ± variance of two animals per group. Two separate experiments are shown for both CD8 responder populations.

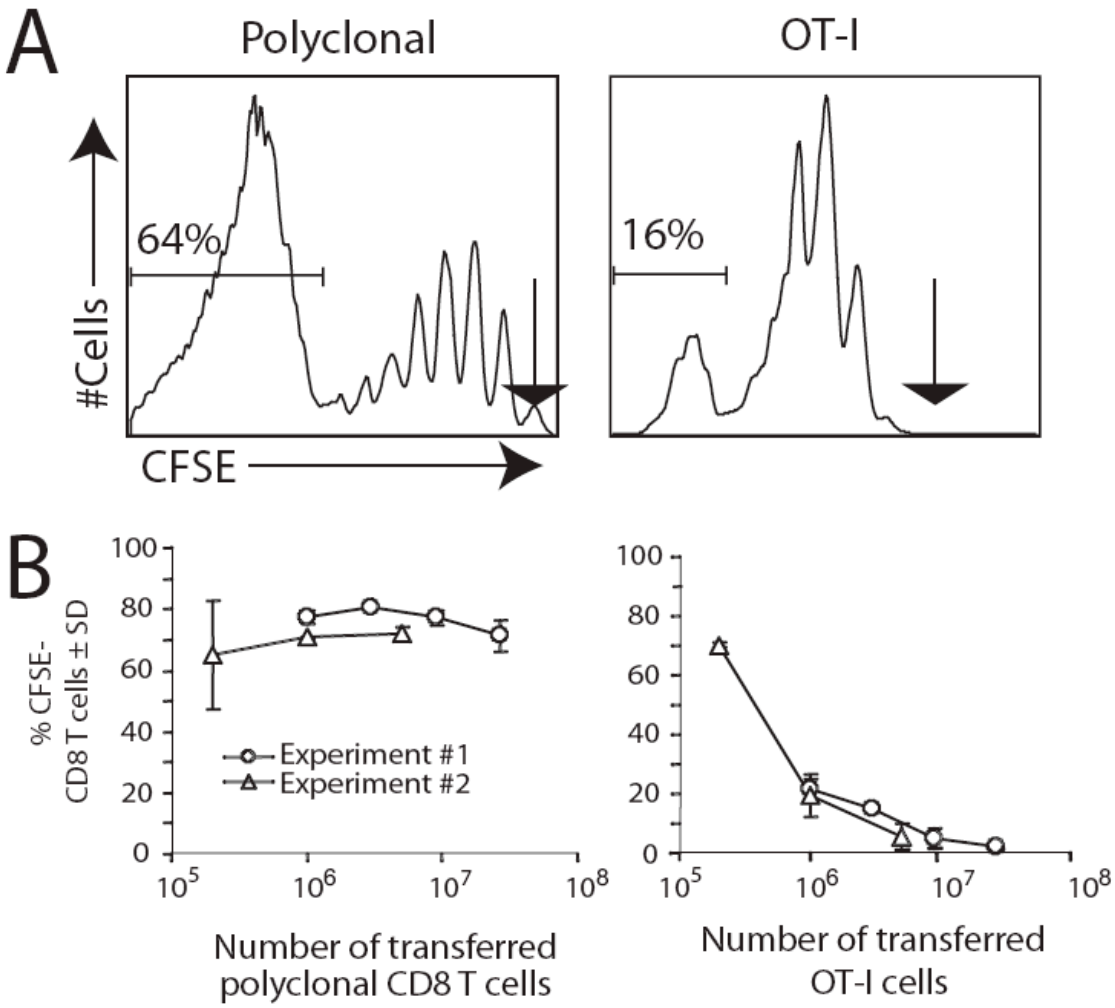

A
Polyclonal
OT-I
64%
16%
#Cells
CFSE
B
% CFSE- CD8 T cells ± SD
Experiment #1
Experiment #2
Number of transferred polyclonal CD8 T cells
Number of transferred OT-I cells

FIGURE 2-2. Tregs selectively suppress the spontaneous form of LIP experienced by naive CD8 T cells. $CD25^+$ cells were injected via tail vein into $RAG^{-/-}$ recipients on day –7 (2 x 10^6 cells/animal). Approximately 1 x 10^7 OT-1 $RAG^{-/-}$ (high transfer) or 1 x 10^6 (low transfer) each of OT-1 or naive polyclonal CD8 T cells were CFSE-labeled and injected on day 0 into the same recipients, $RAG^{-/-}$ mice that had not received $CD25^+$ cells on day –7, or wild-type mice. Lymph nodes were harvested for FACS analysis on day 10. Responder T cells were gated using staining for CD45.1 or Thy1.1 congenic markers and CD8. *A*, Representative CFSE profiles of responding CD8 T cells in the lymph nodes from individual animals. Solid line histograms show CFSE content of CD8 T cells in $RAG^{-/-}$ animals in absence of Tregs. Stippled line histograms show CFSE content of CD8 T cells in $RAG^{-/-}$ animals in the presence of Tregs. Shaded histograms show CFSE content of CD8 T cells transferred into wild-type recipients. Similar results were obtained from more than two independent experiments (two or more individual mice per experiment). *B*, The data for the $RAG^{-/-}$ recipient mice were analyzed for percentage of $CFSE^-$ fractions in different responder CD8 T cell populations, as well as the CFSE content in the $CFSE^+$ CD8 T cells, a measure of overall cell cycle activity in this subpopulation of responders, represented by the mean fluorescence intensity (MFI). Each bar represents the mean ± SD of four animals. Statistical significance was determined using the two-tailed Student's *t* test, comparing the group with pretransfer of Tregs with the control group without pretransfer of Tregs. Stars represent a statistical significance with $p < 0.05$.

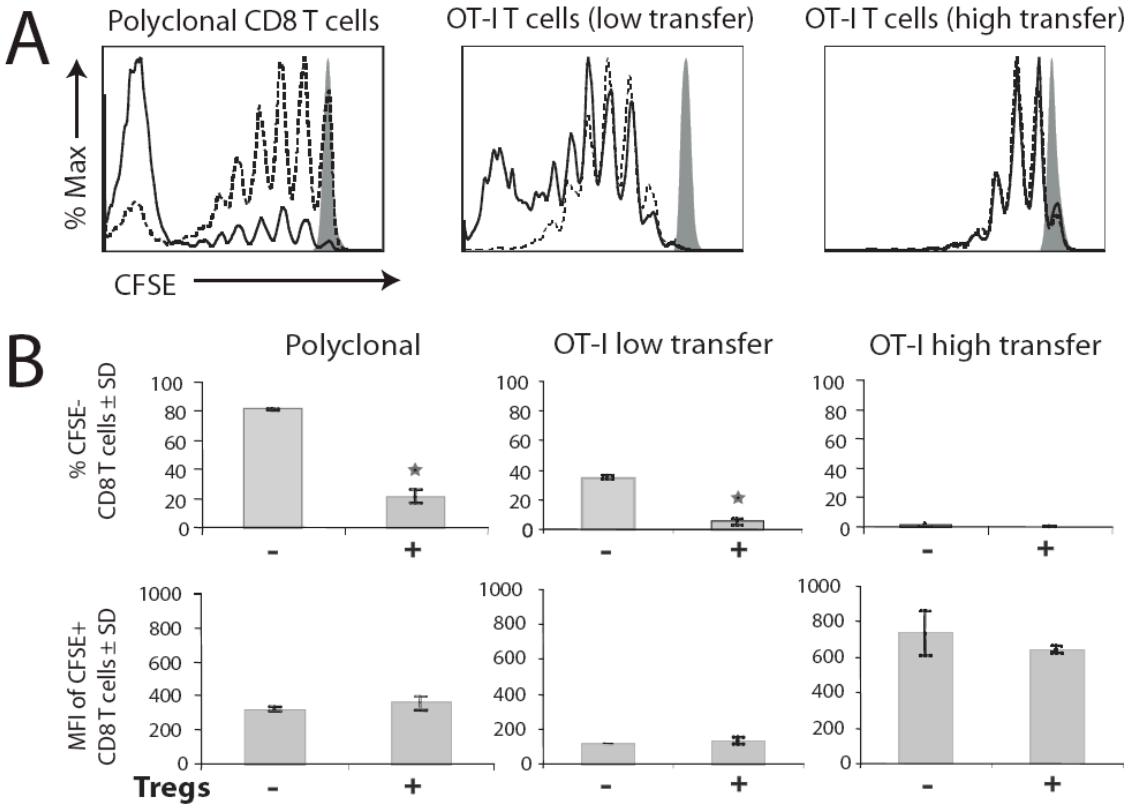

A
Polyclonal CD8 T cells
OT-I T cells (low transfer)
OT-I T cells (high transfer)
% Max
CFSE
B
Polyclonal
OT-I low transfer
OT-I high transfer
% CFSE- CD8 T cells ± SD
MFI of CFSE+ CD8 T cells ± SD
100
80
60
40
20
0
1000
800
600
400
200
Tregs
-
+

FIGURE 2-3. Tregs suppress expression of effector characteristics by CD8 T cells undergoing spontaneous LIP. Approximately 2 x 10^6 $CD25^+$ cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 3 x 10^6 naive polyclonal CD8 T cells were CFSE-labeled and injected on day 0 into the same recipients (or into $RAG^{-/-}$ mice that had not received $CD25^+$ cells on day –7). Tissues were harvested on day 10, stained for granzyme B, and analyzed by FACS. *A*, Each plot is gated on the responder CD8 T cells and represents data from a single animal. The vertical line in each plot represents separation between $CFSE^-$ and $CFSE^+$ responder T cells. Numbers in plots represent percentages of $CFSE^-$ cells in the sample. *B*, Data from two independent experiments were pooled and analyzed for percentage of $CFSE^-$ cells in different responder CD8 T cell populations. CFSE content in the $CFSE^+$ fraction is represented by the MFI. Each bar represents the mean of four animals ± SD. Statistical significance was determined using the two-tailed Student's *t* test. Stars represent a statistical significance with $p < 0.05$. *C*, Absolute number of $CFSE^-$ and $CFSE^+$ responder CD8 T cells in the indicated tissues. Green bars represent the number of responder cells in absence of Tregs, while red bars represent the number of responder cells in the presence of Tregs.

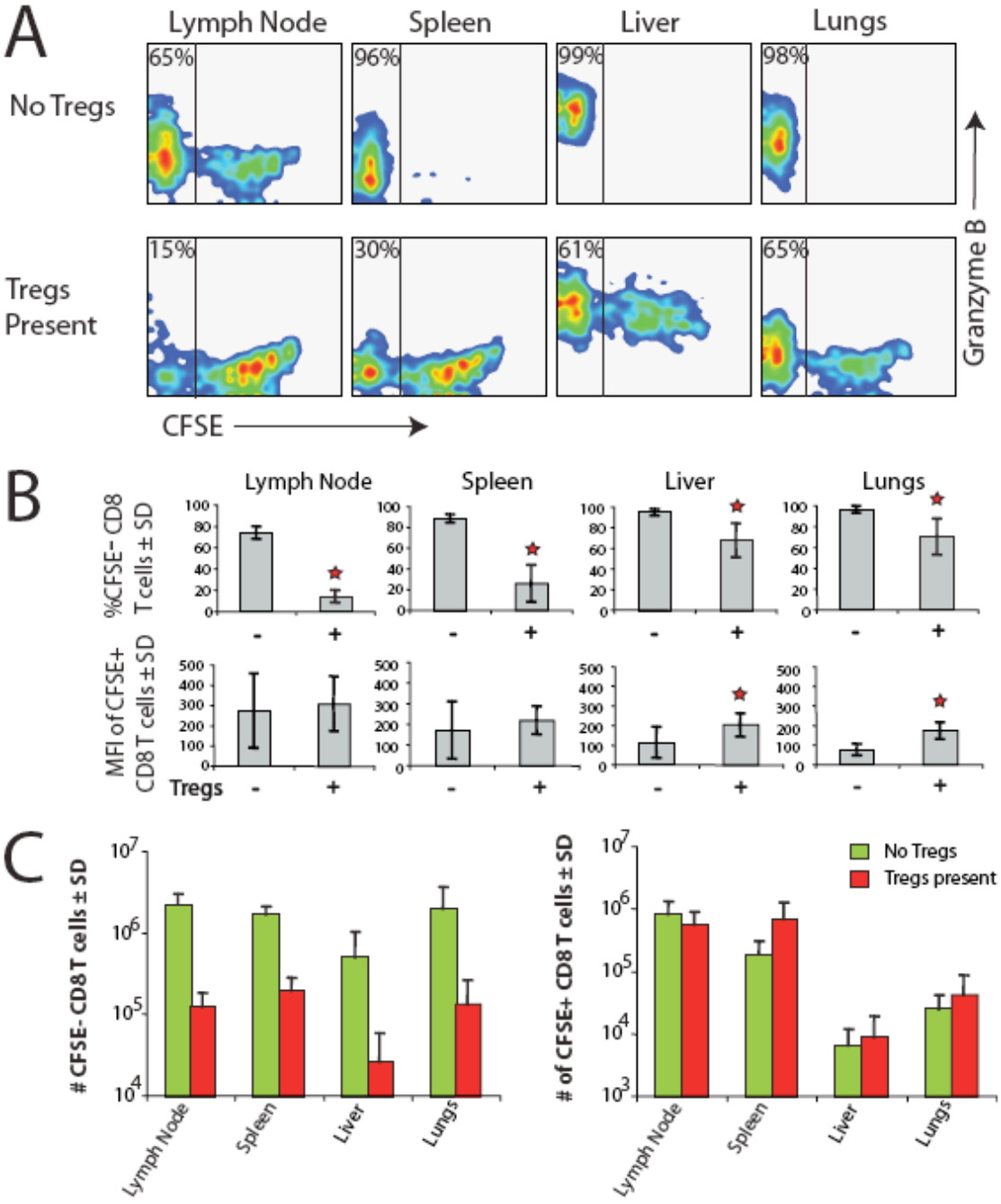

A
Lymph Node
Spleen
Liver
Lungs
No Tregs
65%
96%
99%
98%
Tregs Present
15%
30%
61%
65%
Granzyme B
CFSE
B
Lymph Node
Spleen
Liver
Lungs
%CFSE- CD8 T cells ± SD
MFI of CFSE+ CD8 T cells ± SD
Tregs
C
CFSE- CD8 T cells ± SD
of CFSE+ CD8 T cells ± SD
No Tregs
Tregs present
Lymph Node
Spleen
Liver
Lungs

FIGURE 2-4. $CD4^+CD25^-$ T cells enhance spontaneous LIP of CD8 T cells. Approximately 2.5 x 10^5 naive $CD4^+CD25^-$ T cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 1 x 10^5 OT-1 $RAG^{-/-}$ or naive polyclonal CD8 T cells were CFSE-labeled and injected into the same recipients on day 0 (or into $RAG^{-/-}$ mice that had not received $CD4^+CD25^-$ cells on day –7). Lymph node cells were harvested on day 10 and stained for granzyme B, and analyzed by FACS. *A*, Representative CFSE profiles of responding CD8 T cells in the lymph nodes from individual animals. Histograms are shown for total (*upper panels*) and only the $CFSE^+$ (*lower panels*) responder CD8 T cells. Solid line histograms show CFSE content of responders in $RAG^{-/-}$ animals in the absence of CD4 T cells, and stippled line histograms show CFSE content of responders in $RAG^{-/-}$ animals in the presence of CD4 T cells. *B*, *Left panel*, The CFSE content of CD8 T cells undergoing homeostatic proliferation ($CFSE^+$ cells), represented by the MFI, in the presence or absence of CD4 T cells. *Center panel*, The absolute number of total CD8 T cells in the presence or absence of CD4 T cells. *Right panel*, The fraction of CD8 responders undergoing spontaneous proliferation ($CFSE^-$) (gray bar), and the subfraction of these that express granzyme B (black bar). Each bar represents the mean ± SD of three animals. Statistical significance was determined using the two-tailed Student *t* test comparing groups with pretransfer of CD4 T cells with the control groups without pretransfer of CD4 T cells. Stars represent a statistical significance with $p < 0.05$.

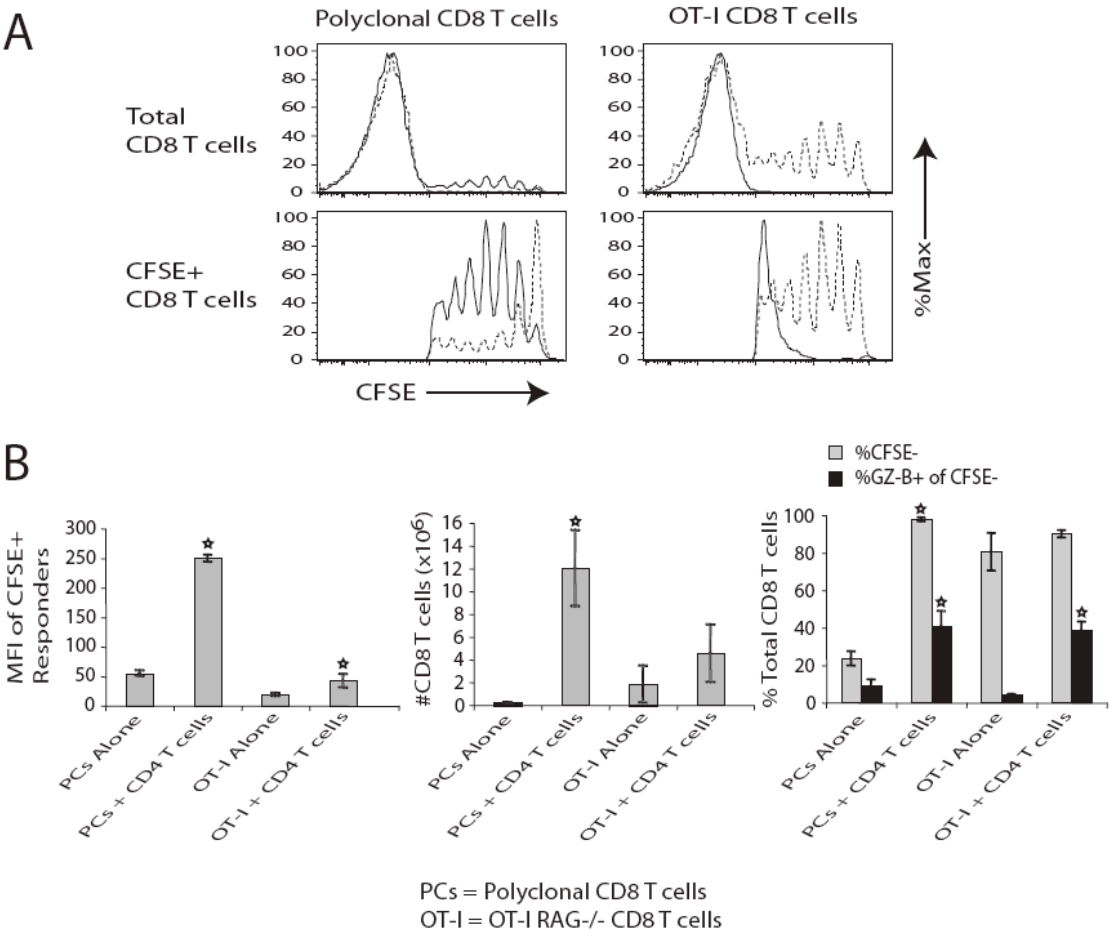

A
Polyclonal CD8 T cells
OT-I CD8 T cells
Total CD8 T cells
CFSE+ CD8 T cells
%Max
CFSE
B
%CFSE-
%GZ-B+ of CFSE-
MFI of CFSE+ Responders
#CD8 T cells (x10^6)
% Total CD8 T cells
PCs Alone
PCs + CD4 T cells
OT-I Alone
OT-I + CD4 T cells
PCs = Polyclonal CD8 T cells
OT-I = OT-I RAG-/- CD8 T cells

FIGURE 2-5. Tregs from young wild-type and IL-10$^{-/-}$ mice are phenotypically similar. *A*, FACS analysis of suspensions of pooled lymph nodes and spleens from four wild-type and four IL-10$^{-/-}$ mice before and after purification by positive selection with anti-CD25 mAb (PC61). Numbers in plots represent gated cells as a percent of total. *B*, Histograms show surface staining for CD69, GITR, and intracellular staining for CTLA-4 in wild-type (blue lines) and IL-10$^{-/-}$ (red lines) Tregs isolated as described in *A*, and Foxp3^{-} CD4 T cells (shaded). *C*, Approximately 6 x 10^5 wild-type or IL-10$^{-/-}$ CD25^{+} cells were CFSE-labeled and injected into RAG$^{-/-}$ recipients on day 0. Lymph nodes were harvested on day 7 and analyzed by FACS. Histograms show representative CFSE profiles of wild-type (blue histograms) and IL-10$^{-/-}$ (red histograms) Tregs transferred into RAG$^{-/-}$ recipients. The bar graph represents absolute numbers of CD4^{+}Foxp3^{+} cells in the lymph nodes (pooled axial, brachial, inguinal, and mesenteric) of RAG$^{-/-}$ animals that received either wild-type or IL-10$^{-/-}$ CD25^{+} cells.

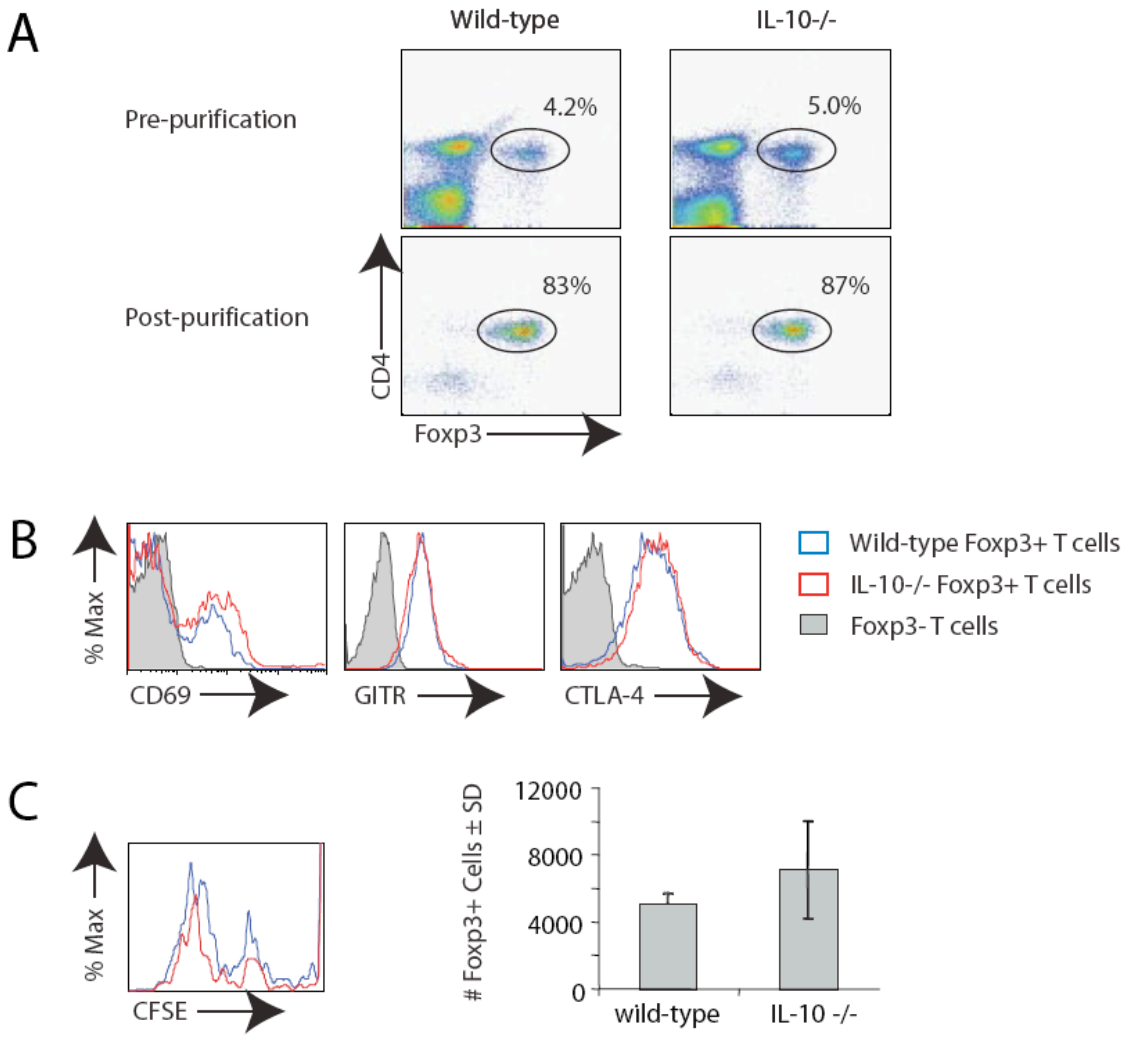

A
Wild-type
IL-10-/-
Pre-purification
Post-purification
4.2%
5.0%
83%
87%
CD4
Foxp3
B
% Max
CD69
GITR
CTLA-4
Wild-type Foxp3+ T cells
IL-10-/- Foxp3+ T cells
Foxp3- T cells
C
% Max
CFSE
Foxp3+ Cells ± SD
12000
8000
4000
0
wild-type
IL-10 -/-

FIGURE 2-6. Suppression of CD8 T cell spontaneous LIP by Tregs is not IL-10 dependent. Approximately 2 x 10^6 wild-type or IL-$10^{-/-}$ $CD25^+$ cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 3 x 10^6 naive polyclonal CD8 T cells were CFSE-labeled and injected on day 0 into the same recipients (or into $RAG^{-/-}$ mice that had not received Treg cells on day –7). Tissues were harvested on day 10 and analyzed by FACS. *A*, Representative CFSE profiles of CD8 responder T cells in indicated groups of mice and tissues. The numbers are percentages of responder CD8 T cells undergoing spontaneous LIP ($CFSE^-$ cells). *B*, Total numbers of CD8 responders recovered from different tissues in indicated groups of mice. Each bar shows the average of four animals pooled from two separate experiments. Stars represent a statistical significance with $p < 0.05$ by Student's two-tailed *t* test when comparing the number of cells isolated from tissues of animals receiving a pretransfer of Tregs with those from animals without pretransfer of Tregs.

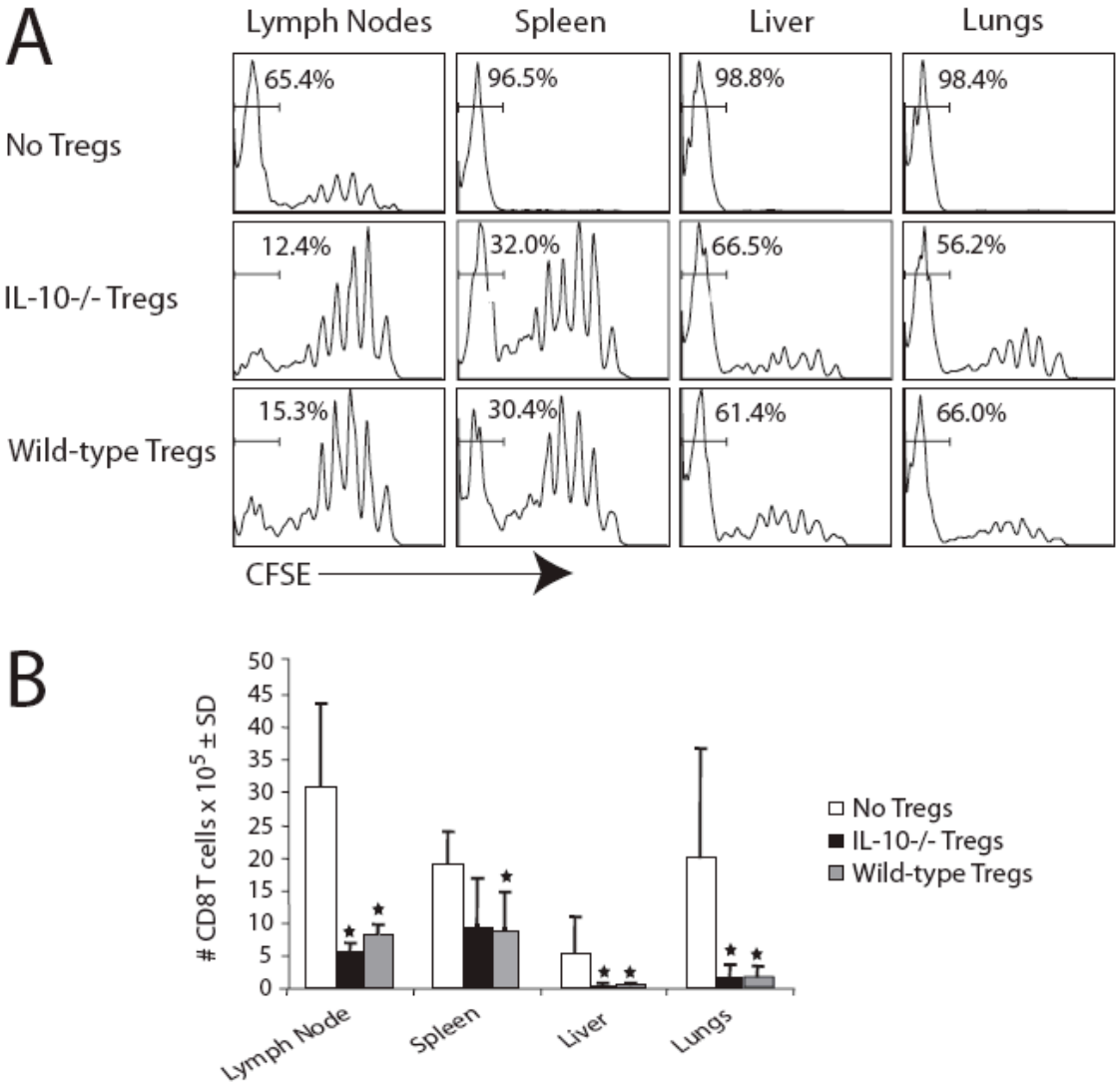

A
Lymph Nodes
Spleen
Liver
Lungs
No Tregs
IL-10-/- Tregs
Wild-type Tregs
65.4%
96.5%
98.8%
98.4%
12.4%
32.0%
66.5%
56.2%
15.3%
30.4%
61.4%
66.0%
CFSE
B
CD8 T cells x 10^5 ± SD
50
45
40
35
30
25
20
15
10
5
0
Lymph Node
Spleen
Liver
Lungs
No Tregs
IL-10-/- Tregs
Wild-type Tregs

FIGURE 2-7. Suppression of CD4 T cell spontaneous LIP by Tregs is not IL-10 dependent. Approximately 5 x 10^5 wild-type or IL-$10^{-/-}$ $CD25^+$ cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 2.5 x 10^6 naive polyclonal CD4 T cells were CFSE-labeled and injected on day 0 into the same recipients (or into $RAG^{-/-}$ mice that had not received Treg cells on day –7). Lymph nodes were harvested on day 7 and analyzed by FACS. *A*, Representative CSFE profiles of responding CD4 T cells in the lymph nodes. Solid line histograms represent CFSE content of CD4 responders in the absence of Tregs. Stippled line histograms represent CFSE content of CD4 responders in the presence of Tregs. *B*, Percentages of $CFSE^-$ responder CD4 T cells in indicated groups of mice, as well as the CFSE content in the $CFSE^+$ CD4 T cell responders represented by the MFI. *C*, Relative numbers of indicated CD4 T cells responders in different groups of mice. Each bar represents the mean ± SD of three animals. Statistical significance was determined using the two-tailed Student *t* test. One star represents a statistical significance with $p < 0.05$; Two stars represent a statistical significance with $p < 0.01$. Skin-draining lymph nodes (LN) include axial, brachial, cervical, and inguinal.

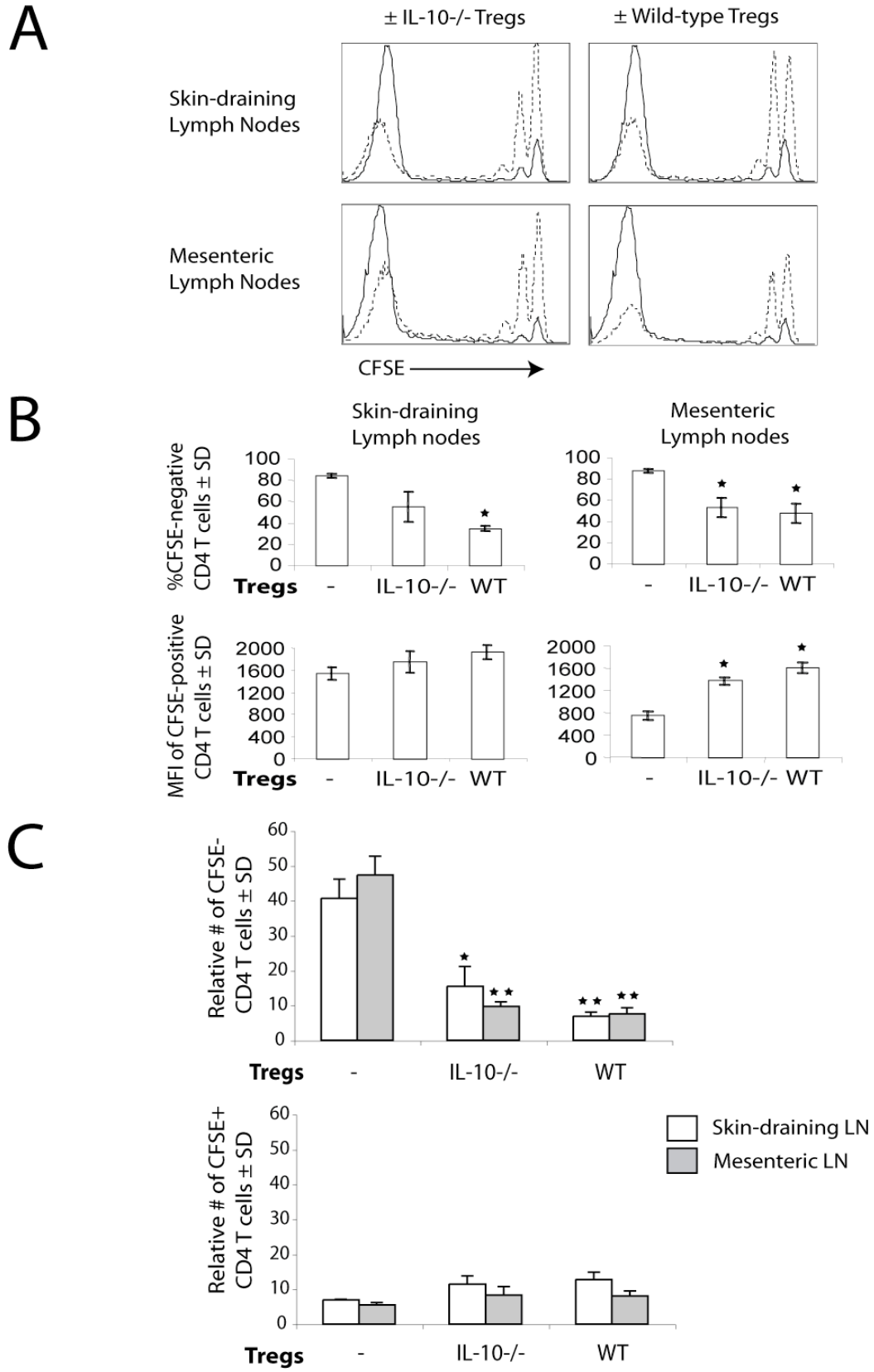
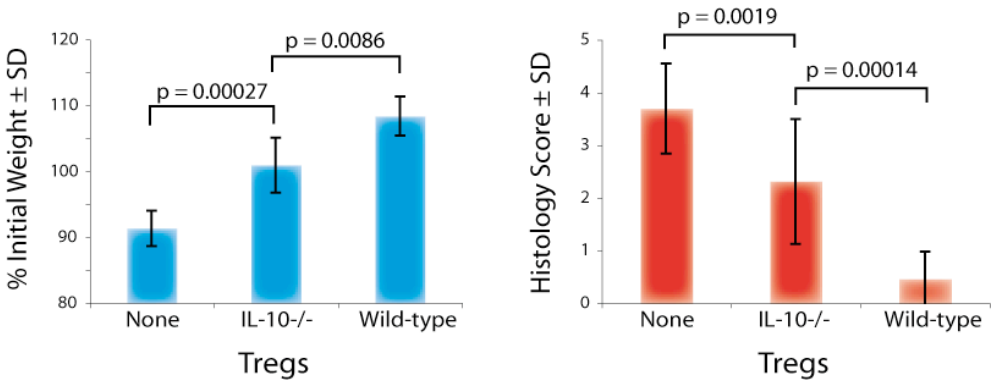

A
± IL-10-/- Tregs
± Wild-type Tregs
Skin-draining Lymph Nodes
Mesenteric Lymph Nodes
CFSE
B
Skin-draining Lymph nodes
Mesenteric Lymph nodes
%CFSE-negative CD4 T cells ± SD
MFI of CFSE-positive CD4 T cells ± SD
Tregs - IL-10-/- WT
C
Relative # of CFSE- CD4 T cells ± SD
Relative # of CFSE+ CD4 T cells ± SD
Skin-draining LN
Mesenteric LN

FIGURE 2-8. IL-10-deficient Tregs cannot completely prevent colitis caused by adoptive transfer of CD4 T cells into $RAG^{-/-}$ mice. Approximately 5 x 10^5 wild-type or IL-$10^{-/-}$ $CD25^+$ cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 1 x 10^5 naive polyclonal CD4 T cells were injected on day 0 into the same recipients (or into $RAG^{-/-}$ mice that had not received Treg cells on day –7). Mice were sacrificed when the group of $RAG^{-/-}$ mice without pretransfer of Tregs was showing obvious clinical signs of disease, including diarrhea and hunched posture. Three separate experiments were harvested 6, 8, or 9 wk after transfer and the results were pooled here. Histologic scores were determined on H&E sections of distal colons blinded to the identity of the experimental groups. *A*, Mean changes ± SD in weight of the animals and histologic scores ± SD in indicated groups of mice. The pooled experiments together totaled 13 mice per group. Statistical significance was determined using the two-tailed Student *t* test. *B*, Representative H&E sections from each of the experimental groups.

A


% Initial Weight ± SD
120
110
100
90
80
p = 0.00027
p = 0.0086
None
IL-10-/-
Wild-type
Tregs
Histology Score ± SD
5
4
3
2
1
0
p = 0.0019
p = 0.00014
None
IL-10-/-
Wild-type
Tregs

B

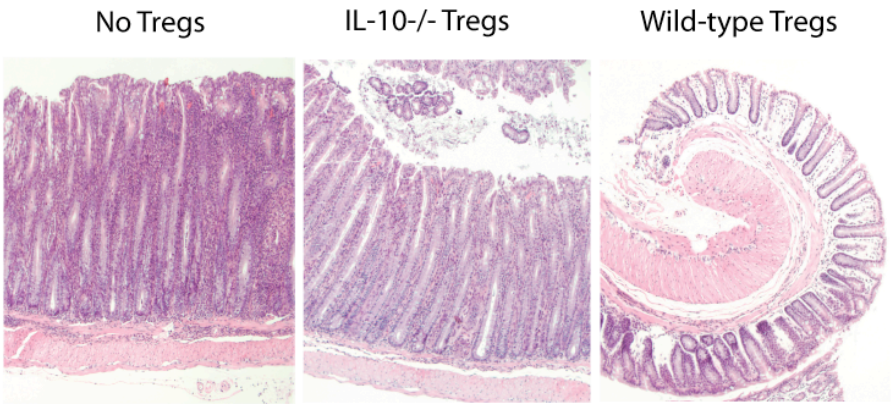
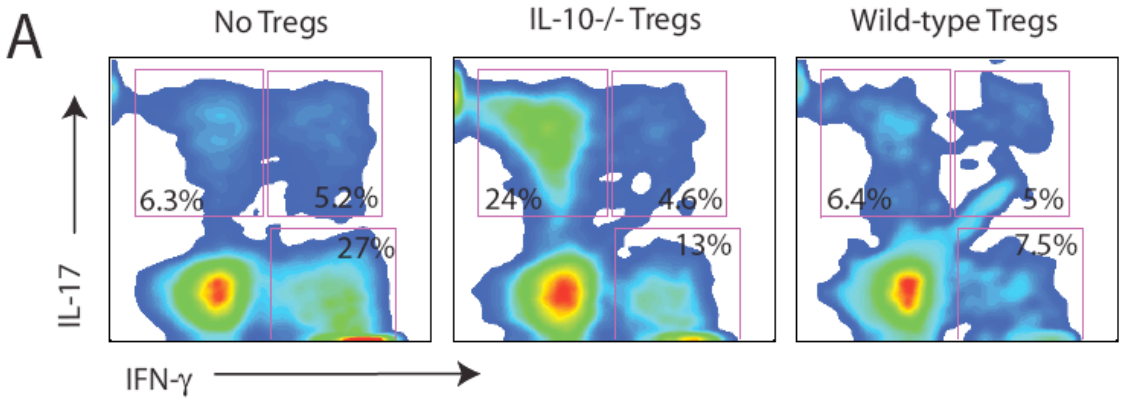

No Tregs
IL-10-/- Tregs
Wild-type Tregs

FIGURE 2-9. IL-10-deficient Tregs are impaired in their ability to suppress effector CD4 T cells in the colon. Approximately 5 x 10^5 wild-type or IL-$10^{-/-}$ $CD4^+CD25^+$ T cells were transferred into $RAG^{-/-}$ recipients on day –7. Approximately 1 x 10^5 naive polyclonal CD4 T cells were injected on day 0 into the same recipients (or into $RAG^{-/-}$ mice that had not received Treg cells on day –7). Mice were sacrificed at 9 wk, and cells isolated from the lymph nodes and colons were stimulated with PMA/ionomycin in vitro, stained for cytokine expression, and subjected to FACS analysis. *A*, Representative FACS plots of gated CD4 T cells in the mesenteric lymph nodes of individual animals. Percent numbers show fractions of total CD4 T cells expressing indicated cytokines. *B*, Number of effector cytokine-producing CD4 T cells in mesenteric lymph nodes and colons. Relative CD4 T cell numbers were calculated in comparison to the group that did not receive a pretransfer of Tregs. Each bar represents the mean ± SD of at least six animals. Statistical significance was determined using the two-tailed Student *t* test. The black and red stars indicate statistical significance with $p < 0.05$ and $p < 0.01$, respectively. WT, Wild type.



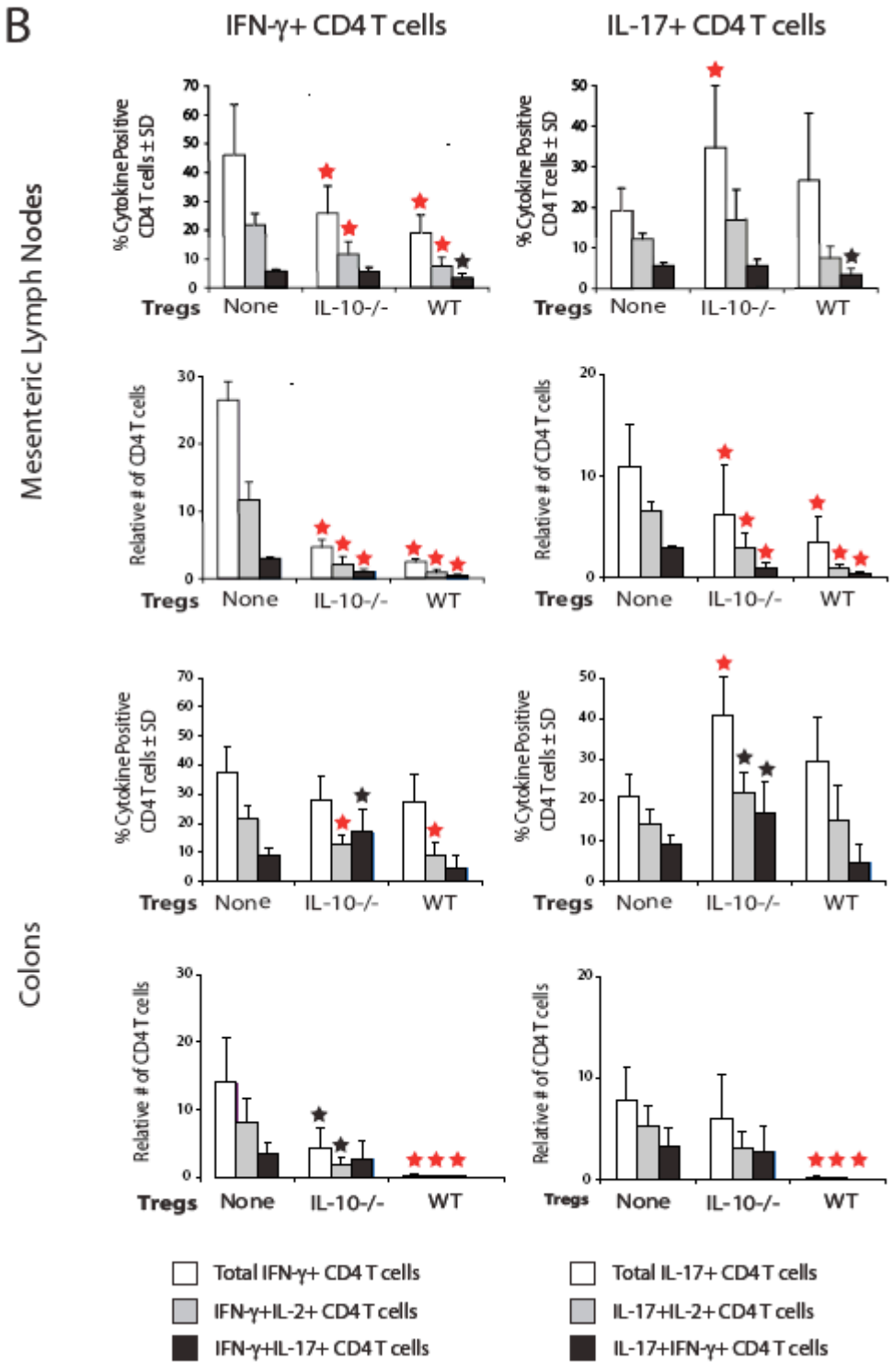

B
IFN-γ+ CD4 T cells
IL-17+ CD4 T cells
Mesenteric Lymph Nodes
Colons
% Cytokine Positive CD4 T cells ± SD
Relative # of CD4 T cells
Tregs
None
IL-10-/-
WT
Total IFN-γ+ CD4 T cells
IFN-γ+IL-2+ CD4 T cells
IFN-γ+IL-17+ CD4 T cells
Total IL-17+ CD4 T cells
IL-17+IL-2+ CD4 T cells
IL-17+IFN-γ+ CD4 T cells

FIGURE 2-10. When co-transferred, large numbers of Tregs are required to suppress infiltration of effector CD4 T cells in the colon. In one set of mice, indicated numbers of wild-type or IL-10$^{-/-}$ CD4^{+}CD25^{+} T cells were transferred into RAG$^{-/-}$ recipients on day –7, then 1 x 10^{5} naive polyclonal CD4 T cells were injected into the same recipients on day 0. In a second set of mice, indicated numbers of wild-type or IL-10$^{-/-}$ CD4^{+}CD25^{+} T cells were co-transferred with 1 x 10^{5} naive polyclonal CD4 T cells on day 0. Control RAG$^{-/-}$ mice received only 1 x 10^{5} naive CD4 T cell responders on day 0. Mice were sacrificed at 12 wk following adoptive transfer of CD4 T cell responders. *A*, Mean changes in weight of the animals and histologic scores for colon sections for the indicated groups of mice. Histologic scores were determined on H&E sections of distal colons blinded to the identity of the experimental groups. Each bar represents the mean ± SEM of four animals. *B*, Relative CD4 responder T cell and Treg numbers were calculated based on total lymphocyte numbers. WT, Wild type; MLN, mesenteric lymph nodes.

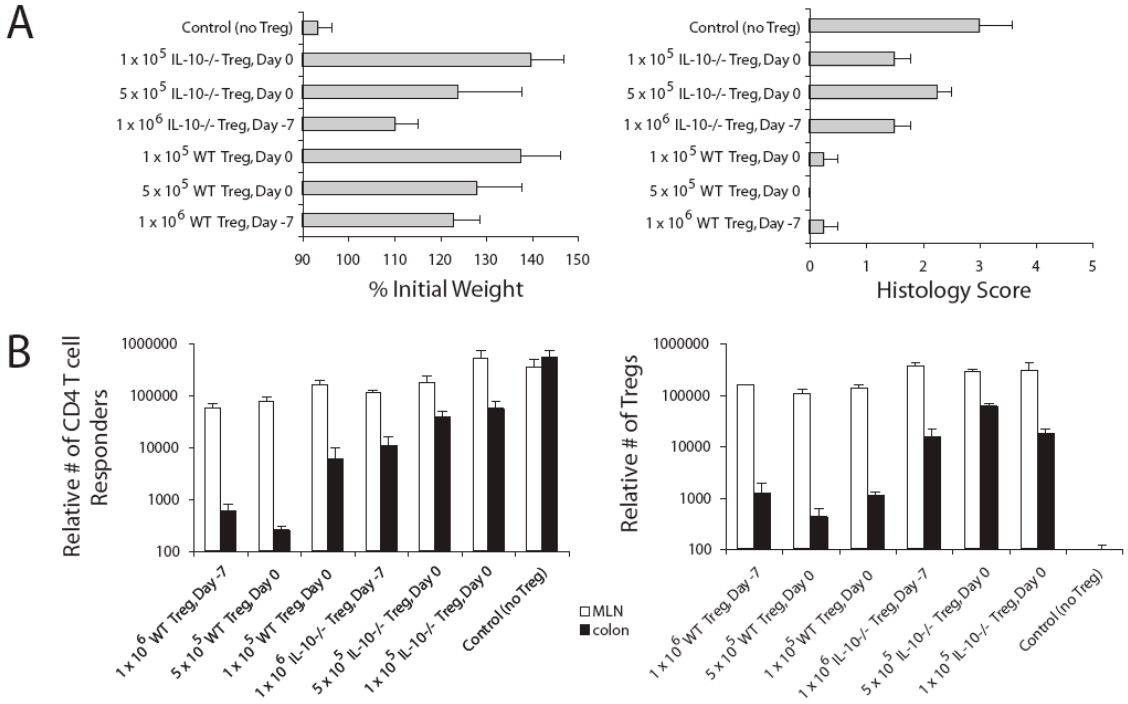
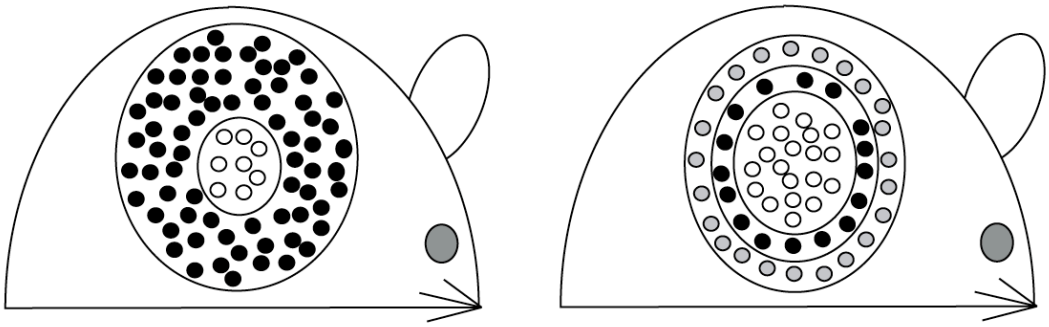
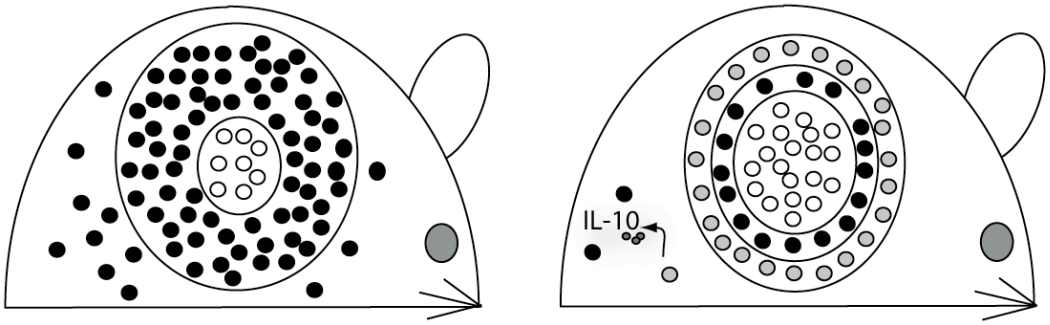

A
Control (no Treg)
1 x 10^5 IL-10-/- Treg, Day 0
5 x 10^5 IL-10-/- Treg, Day 0
1 x 10^6 IL-10-/- Treg, Day -7
1 x 10^5 WT Treg, Day 0
5 x 10^5 WT Treg, Day 0
1 x 10^6 WT Treg, Day -7
90 100 110 120 130 140 150
% Initial Weight
0 1 2 3 4 5
Histology Score
B
Relative # of CD4 T cell Responders
1000000
100000
10000
1000
100
MLN
colon
1 x 10^6 WT Treg, Day -7
5 x 10^5 WT Treg, Day 0
1 x 10^5 WT Treg, Day 0
1 x 10^6 IL-10-/- Treg, Day -7
5 x 10^5 IL-10-/- Treg, Day 0
1 x 10^5 IL-10-/- Treg, Day 0
Control (no Treg)
Relative # of Tregs

FIGURE 2-11. Model of Treg-mediated control of responder T cells in lymphopenia. The *top half* of the figure illustrates control of LIP by Tregs within the secondary lymphoid tissues. Responder T cells and Tregs are shown to exist in different compartments, although it is possible that Tregs (shaded circles) and responder T cells (black circles) undergoing spontaneous LIP compete for some common resources. The presence of Tregs limits spontaneous LIP, which in turn limits the number of potentially autoreactive T cells capable of causing autoimmunity. This function of Tregs is independent of their ability to produce IL-10. The *bottom half* of the figure illustrates the second phase of Treg-mediated suppression, which happens in the periphery. Some effector T cells generated in the course of spontaneous LIP leave the lymphoid tissues. However, their local activity and ability to trigger inflammation in the periphery is inhibited by Tregs in an IL-10-dependent manner. Finally, we speculate at this time that T cells undergoing spontaneous proliferation compete for resources with T cells undergoing homeostatic proliferation (white circles). Therefore, uncontrolled spontaneous LIP may limit homeostatic proliferation and potentially impair overall TCR diversity during immune reconstitution.

1. Tregs restrain spontaneous LIP in lymphoid tissues independently of IL-10



2. Treg-mediated suppression of pathogenic effectors in the periphery is dependent on IL-10



- ● T cells undergoing spontaneous LIP
- ○ T cells undergoing homeostatic LIP
- ○ Regulatory T cells (Tregs)

Chapter 3

CD4+CD25+Foxp3+ regulatory T cells maintain optimal responsiveness of the Foxp3- T cell population during reconstitution from lymphopenia

Abstract

T cell diversity is a key feature of the adaptive immune system. Lymphopenia-induced proliferation (LIP) can trigger oligoclonal expansion of T cells reacting to foreign or self-antigens. This can result in loss of T cell diversity and compromise immune fitness that can be achieved following reconstitution by LIP. We have demonstrated that CD4+CD25+Fopx3+ regulatory T cells (Tregs) selectively inhibit the spontaneous (fast) form of LIP, which is associated with differentiation into memory/effector-like cells, and is likely to contain the most reactive T cell clones. Selective suppression of these clones is likely to leave more resources for T cells undergoing the slower homeostatic form of LIP. We hypothesize, therefore, that one of the functions of Tregs is to maintain the T cell diversity and overall immune fitness during reconstitution from lymphopenia. Structural data generated using flow cytometric analysis of TCR Vβ usage and PCR-based TCR Jβ spectratyping suggest Tregs effectively prevent the emergence of 'holes' in the T cell repertoire following LIP. Furthermore, using the *Listeria monocytogenes* infection model and magnetic bead enrichment with specific MHC class I and II tetramers, we demonstrate that the presence of Tregs during immune reconstitution allows for more accumulation of pathogen-associated antigen-specific T cells in secondary lymphoid tissues following clearance of the bacteria. This result may otherwise seem paradoxical as Tregs are typically thought of as generalized suppressors of the immune system. We believe these results provide some insight into the central role of Tregs in the immune system, vaccine responsiveness, pathogenesis of autoimmunity, and immune senescence.

Introduction

Maintenance of peripheral T cell homeostasis is a critical feature of the adaptive immune system (82). Strict control of T cell homeostasis ensures adequate peripheral cell numbers, T cell receptor (TCR) diversity, responsiveness to foreign antigens, and self-tolerance. If a state of peripheral T cell deficiency is created, the residual T cells are driven to undergo proliferation by a process termed lymphopenia-induced proliferation (LIP) to reconstitute the optimal T cell numbers. LIP can be observed in a number of physiological and pathological situations (81). Lymphopenia is physiologic during the prenatal and neonatal periods, and LIP may contribute to generation of sufficient numbers of T cells with a memory phenotype (79). In addition, as the thymus involutes with increasing age, LIP assumes greater importance in maintaining T cell population size (80). Transient lymphopenia can accompany virtually any viral infection, and some viral infections, e.g., HIV, lead to chronic and progressive lymphopenia. In addition, there are multiple iatrogenic causes of lymphopenia, including cytodepletion by radiation, chemotherapy, and depleting antibodies. Notably, lymphopenia can be a trigger to autoimmunity, which is at least in part explained by T cell resistance to tolerance induction during LIP (83). Furthermore, as individual T cell clones expand at different rates during LIP, the emerging T cell population risks loss of TCR diversity and diminished immune fitness. However, although lymphopenic states occur multiple times during lifetimes of all individuals, autoimmunity and immune deficiency do not manifest in most people. Therefore, there must be mechanisms that help to maintain optimal immunologic tolerance and immune fitness during immune reconstitution.

Regulatory $CD4^+CD25^+Foxp3^+$ T cells (Tregs) constitute 5-15% of peripheral CD4 T cells in healthy, adult mice and humans (40), and are critical in the maintenance of immunologic tolerance and peripheral T cell homeostasis. These cells are able to suppress a range of immunologic responses in vitro and in vivo. Their discovery was aided by a number of animal models of autoimmunity associated with lymphopenia (neonatal thymectomy, the SCID adoptive transfer colitis model, and autoimmune thyroiditis) (79, 97, 100). However, early studies failed to demonstrate their ability to suppress LIP. Since then at least two major forms of LIP were recognized – homeostatic and spontaneous (89). Homeostatic LIP is driven primarily by cytokines such as IL-7 and IL-15, and is generally slow and steady. Spontaneous LIP is burst-like and is likely driven by cognate antigen stimulation, and is the most likely source of pathogenic T cells with higher TCR affinity for self- and commensal flora antigens. We have shown previously that while Tregs do not suppress homeostatic LIP, they do suppress the spontaneous form of LIP (160). This likely contributes to their protective function against development of autoimmunity during LIP. We also thought it was possible that selective suppression of spontaneous LIP could benefit T cells with lower TCR affinity for self antigens by allowing greater access to cytokine resources important for their survival and homeostatic LIP. Therefore, we predicted that presence of Tregs during immune reconstitution by LIP should lead to preservation of greater diversity of TCR repertoire, and consequently, better immune fitness.

Here, we characterized the TCR repertoire in T cell populations expanded by LIP in the presence or absence of Tregs following adoptive transfer into recombinase activating gene-deficient (RAG-/-) or TCRα-/- recipients. Using TCR Vβ CDR3

spectratyping we demonstrated significantly enhanced preservation of structural TCR diversity in the presence of Tregs during LIP. The reduction of TCR diversity in absence of Tregs resulted in development of functional 'holes' in the repertoire that were revealed by enumerating antigen-specific T cells following infection with *Listeria monocytogenes*. Thus, markedly greater numbers of *Listeria*-specific responding T cells were found if Tregs were present than if they were absent during LIP. These findings suggest that Tregs preserve TCR diversity and immune responsiveness during immune reconstitution from lymphopenia. This positive effect of Tregs on immunity contrasts with their generally accepted immunosuppressive function.

Materials and Methods

Mice

C57BL6 (B6) and CD45.1 congenic mice were purchased from the National Cancer Institute (Frederick, MD). Some B6 mice used were wild-type littermates to IL-$10^{-/-}$ originally obtained from The Jackson Laboratory (Bar Harbor, ME) and CTLA-$4^{+/-}$ mice, which were a generous gift from Dr. J. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Recombinase gene 1-deficient (RAG-$1^{-/-}$), T-cell receptor alpha chain-deficient (TCR$\alpha^{-/-}$), and Thy1.1 congenic on the B6 background were obtained from Jackson Labs. B6 RAG-$1^{-/-}$ were bred onto the CD45.1 congenic background in our facility. Thy1.1 BALB/c mice were originally provided by Dr. L. Turka (University of Pennsylvania, Philadelphia, PA). RAG-$2^{-/-}$ BALB/c mice were purchased from NCI and bred onto the congenic Thy1.1 background in our facility. All

mice used were generally 4-20 weeks of age. All animals were maintained in a specific pathogen-free facility in microisolator cages with filtered air according to the National Institutes of Health guidelines.

Cell Isolation, labeling, adoptive transfer, and antibody-mediated in vivo depletion

Donor T cells were collected from secondary lymphoid tissues (axillary, brachial, cervical, mesenteric, and inguinal lymph nodes, and spleen). $CD44^{low}$ CD4, CD8, and bulk (both CD4 and CD8) T cells were purified in two stages—first, CD8 or CD4 T cells were prepared by negative selection against CD8 or CD4, MHC class II, CD11b, B220, CD25, and in some cases CD103 (all Abs labeled with FITC) using anti-FITC BioMag particles (Polysciences, PA), followed by depletion of $CD44^{high}$ cells (B6 only) using magnetic microbeads (Miltenyi Biotec, CA), as previously described (123). Briefly, the purified T cells were suspended in labeling buffer (2% FBS in PBS), incubated with 0.004 μg anti-CD44-FITC (eBioscience, CA) per 10^6 cells for 20 minutes, washed, and labeled with anti-FITC magnetic microbeads. The negative fraction was collected following Miltenyi Biotec magnetic column separation. $CD25^+$ T cells were prepared using positive selection with anti-CD25 biotinylated mAb, PC61, and streptavidin-labeled magnetic microbeads (Miltenyi). Purified cells were transferred to $RAG^{-/-}$ or $TCR\alpha^{-/-}$ hosts via intravenous (i.v.) tail injection. In some cases cells were labeled with 7uM CFSE or SNARF-1 prior to transfer. Regulatory T cells were depleted *in vivo* using a single i.p. injection of 400-450 μg anti-Thy1.1 mAb (clone 1A14).

TCR Vβ and Jβ spectratyping

Regulatory and conventional T cells collected from secondary lymphoid tissues were MACS sorted by positive selection for Thy1.1, as described above. Total RNA was extracted from positive (Thy1.1+/Treg) and negative (Thy1.1-/Tconv) fractions with an RNeasy Mini Kit (Qiagen, CA), and first-strand cDNA was generated from total RNA with Oligo$(dT)_{20}$ and SuperScript III reverse transcriptase (Invitrogen, CA). Each cDNA sample was checked for integrity and for detectable TCR Cβ chain using β actin and Cβ-specific primers (5' Cβ1A; 3' Cβ3C) under PCR amplification conditions described below (161). The CDR3 size distribution PCR assay was performed as described (23) (control and spectratyping primers listed in supplemental Table 3-1). Briefly, each TCR Vβ was amplified with Vβ8.1, 8.3 and/or Vβ10-specific sense primers and a Cβ antisense primer (162). For generation of Vβ spectratypes, the Cβ antisense primer was labeled with 6-fluorescein phosphoramidite (6-FAM) on the 5' end. One ul of the previous synthesis, corresponding to the reverse transcription of 0.05-1ug of total RNA was used in the amplification. cDNA was added to tubes already containing a mixture of Taq Master Mix (Qiagen), DNAse/RNAse-free water, Vβ-specific and Cβ-specific primers (10-20uM) and amplification was performed as follows: thirty cycles of 95^{o}C for 1 min/52^{o}C for 1 min/72^{o}C for 1 min, followed by 5 min at 72^{o}C. A second, nested amplification for specific Jβ sequences was performed using one ul of Vβ-specific product. Cycling conditions remained the same, with the exceptions of Master Mix containing 5' 6-FAM – labeled Jβ-specific sense primers and twenty cycles of amplification. The size distribution of each fluorescent PCR product was determined by electrophoresis on an Applied Biosystems 3130xl Genetic Analyzer

(Foster City, CA), and data were analyzed using Genoprofiler 2.1 (163, 164).

TCR Spectratyping Complexity Scores

To assess each individual sample diversity profile as normal or limited, we used a previously reported complexity scoring system (165); that is, a complexity score = (sum of all the peak areas/sum of the major peak areas) x (number of the major peaks). Major peaks were defined as those on the spectratype histogram whose area was at least 10% of the sum of all the peak areas.

Bacterial infections and peptide:MHC tetramer-based enrichment protocol

Wild-type B6 mice were immunized intravenously with approximately $1x10^7$ CFU of a ΔActA strain of *L. monocytogenes* transformed by a plasmid containing ovalbumin and an I-A^b-specific mutant epitope of I-Eα ('2W1S' - courtesy of Dr. SingSing Way at the U. of Minnesota) (166). Bacteria were grown in LB media with 24 ug/ml of chloramphenicol to an absorbance at 600 nm of about 0.1. The actual number of bacteria injected was confirmed by dilution and growth on LB agar plates containing chloramphenicol. K^b-OVA and I-A^b-2W1S tetramers, respectively, were produced in the laboratories of Drs. Stephen Jameson and Marc Jenkins, respectively, as previously described (26, 167). Lymph nodes and spleens were harvested together and processed for K^b-OVA and I-A^b-2W1S double-tetramer enrichment according to the protocol established by Moon et al. (26, 168).

FACS analysis

Mice were sacrificed with CO_2 before spleen and lymph nodes were removed. All secondary lymphoid tissues were disrupted by mashing with a syringe. In some cases, complete media (10% FBS) was supplemented with 10 μg ml^{-1} brefeldin A (Sigma, MO) to prevent lymphokine secretion. Fixation and intracellular staining for cytokines was done following 2-3 hours in vivo challenge with approximately $5x10^7$ CFU LM-2W1S-OVA. All antibodies used for cell surface staining, negative selection, and tetramer enrichment were purchased from eBioscience, with the exceptions of anti-B220 and anti-CD11c (Biolegend, CA), anti-CD8a (Invitrogen), and anti-CD4 single-stain controls (BD Biosciences, CA). Specific T cell subsets were identified using fluorochrome-labeled antibodies against a panel of TCR Vβ receptors (15 in total), CD3e, CD4, CD8, CD44, and congenic markers such as anti-Thy1.1/Thy1.2 and anti-CD45.1/CD45.2. Anti-IFN-γ antibodies were purchased from eBioscience. Staining for Foxp3 was done using an eBioscience kit and instructions provided by the manufacturer. Absolute numbers of T cells were calculated using PKH reference beads (Sigma-Aldrich). Relative numbers of T cells of specific subtype were calculated in comparison to the number of total T cells in the group that did not receive Tregs. All flow cytometry data was acquired on a FACS Calibur, an LSR, or an LSRII (BD Immunocytometry Systems, CA) and analyzed with FlowJo software (Tree Star, OR).

Statistical analysis

All error bars represent the standard error of the mean (SEM), unless noted otherwise. For data in Figures 2, 3, and 5, a two-tailed, paired Student's t test was used for

assessment of the differences between groups. Prism (Graphpad Software, La Jolla, CA) was used for graphs and statistical analysis. Differences were considered to be significant when the p value was less than 0.05.

Results

T cell adoptive transfer system for studies of the TCR repertoire during LIP.

Tregs are well established in their ability to prevent and treat immunopathology arising in the course of immune reconstitution from lymphopenia by suppression of emergence and reactivity of pathogenic T cells. Here, we set out to test the idea that another function of Tregs during immune reconstitution is preservation of immune fitness by maintaining optimal structural TCR diversity of the residual T cell population. In order to establish LIP we used the RAG-/- adoptive transfer model. First, we titrated numbers of bulk, $CD44^{lo}CD25^{-}$ T cells transferred on day 0 and measured the number of T cells expressing each TCR Vβ chain on days 7 and 30 (Fig. 3-1). Similarly to what has been reported previously (91), we found that the ultimate number of T cells in the lymphoid periphery is independent of the initial input cell number. The T cell population size at 30 days was the same for inputs of 10^5 - 10^7 T cells, and it was still rising following adoptive transfer of 10^4 T cells. We decided to exclude 10^4-10^5 T cell transfers from future experiments because any potential TCR repertoire that could emerge from these would by definition be severely limited. Furthermore, adoptive transfer of very low numbers of T cells is associated with development of immunopathology (85, 91). We also excluded 10^7 T cell transfers because these filled the T cell niche within a mere 7 days, and we didn't think that

significant distortion of the TCR repertoire could be established to allow testing of our hypothesis. We chose adoptive transfer of 10^6 T cells as optimal for our experiments because more than one week was required to reach the T cell population plateau, and only mild immunopathology manifesting as dermatitis was noted with this input number after one month following adoptive transfer. Colitis was not detected clinically or by histopathology, in agreement with previous reports (85). In addition, the T cell populations emerging from 10^6 T cell transfers were indistinguishable from the T cell populations derived from 10^7 T cell transfers at least by rough diversity measures such as Vβ repertoire usage.

In order to test the effects of Tregs on the responder T cell population during LIP, we constructed the following experimental system (Fig. 3-2A). First, 10^6 CD25+Tregs are purified from Thy1.1 BALB/c mice and transferred into RAG-/- recipients and allowed to expand for 7-10 days, after which 10^6 Thy1.2 $CD44^{lo}CD25^-$ responder T cells are adoptively transferred. The lymphoid tissues were harvested for T cell population analysis 30 days after the responder T cell transfer. We used this protocol previously to show that Tregs fill their own T cell niche and selectively suppress only the spontaneous form of LIP by the responder T cells.

The TCR Vβ usage of the reconstituted population was similar to the initial population regardless of the presence or absence of Tregs in the host (Fig. 3-2B). Although simple and robust, flow cytometric measurements of TCR Vβ usage are relatively insensitive, in that they do not allow determination of clonal dominance or restriction within populations of cells expressing the same Vβ chain. In order to probe the TCR repertoire further, we generated spectratypes at the level of a particular Vβ

chain, e.g., Vβ10 shown in Fig. 3-2C. This assay did suggest that CD4 T cells undergoing LIP in absence of Tregs do experience contraction in TCR diversity compared to that measured before LIP or after LIP in presence of Tregs. However, we could not detect decreased TCR diversity when CD8 T cells or unfractionated CD25- responder T cells were allowed to undergo LIP under the same conditions.

Presence of Tregs during immune reconstitution protects structural diversity of the responder T cell population.

Although Vβ spectratyping does reflect some level of structural TCR diversity within a T cell population, it isn't particularly sensitive because it is not focused on the hypervariable regions of the TCR. In contrast, TCR Vβ-Jβ spectratyping is based on measurement of complementarity-determining region 3 (CDR3) size distribution within a population of T cells, and provides a more accurate reflection of its clonality. We performed TCR Vβ10 Jβ spectratyping of CD25- responder T cells that had undergone LIP in the presence or absence of Tregs. We measured spectratypes of CD4 T cells, CD8 T cells, and unfractionated T cells (bulk) under these conditions. In all cases the diversity of the TCR repertoire became severely constricted if Tregs were absent during LIP (Fig. 3-3A). Visual inspection of spectratyping histograms from several TCR Vβ families also allowed us to visualize actual 'holes' in the reconstituted repertoire, as defined by specific Jβ chain representation below our spectratyping limit of detection (approximately 10,000 cells) (Fig. 3-3B). These 'holes' appeared only if Tregs were absent during LIP. In contrast, relative preservation of TCR complexity was noted if Tregs were present during LIP (Fig. 3-3B).

Presence of Tregs during immune reconstitution prevents emergence of repertoire 'holes' that can limit antigen-specific responsiveness to microbial pathogens.

The value of a diverse, polyclonal T cell repertoire is well appreciated in the context of infection by a microbial pathogen. Therefore, we wished to test the biological significance of LIP-associated TCR repertoire constriction in a model of infectious disease such as Listeriosis (infection with the pathogenic bacteria Listeria monocytogenes).

Extensive TCR sequence analysis by Casrouge and colleagues demonstrated that in steady state a mouse spleen contains about 2×10^6 distinct naïve αβ TCR clones. Each T cell clone might be able to recognize more than one antigen, and it has been estimated that lymphoid tissues of a mouse contain 50-500 distinct T cell clones in the naïve T cell repertoire able to recognize a specific peptide/MHC epitope (26, 169). Work published by Moon et al. using tetramer-based enrichment of epitope-specific T cells estimated the number of IA^b-2W1S-specific $CD4^+$ cells in a naïve C57BL/6 mouse to be ≥200 (26). Using the same basic methodology, the naïve (precursor) frequency of K^b-OVA-specific $CD8^+$ cells was found by Obar et al. to be approximately 100 cells per mouse (28). One would, therefore, expect that adoptive transfer of 1×10^6 naïve donor T cells into RAG-/- or TCRα-/- recipients should introduce approximately 2-4 T cells with specificity for K^b-OVA and IA^b-2W1S, respectively. Our hypothesis, however, predicts that the fate of these naïve precursor cells during LIP may be different depending on the presence or absence of Tregs. Specifically, we thought that since these T cells have foreign antigen specificity, they would be less likely to undergo

spontaneous LIP, and would, in fact, benefit from suppression of spontaneous LIP by Tregs.

In order to test the hypothesis, we reconstituted RAG-/- or TCRα-/- mice by adoptive transfer of Thy1.2 CD25-CD44^{low} CD4 T cells (Fig. 3-4A). Some of the recipient animals were pre-transferred with Thy1.1 CD25+ Tregs, and some were not. After 30 days of reconstitution the mice were infected with attenuated *L. monocytogenes* bacteria expressing 2W1S and OVA epitopes. The variable we wanted to test was presence or absence of Tregs *during* LIP. Obviously, presence or absence of Tregs during the infection could also alter the T cell response. Therefore, we added two additional experimental groups. Some of the mice that received Tregs before the adoptive transfer of naïve responder T cells were treated with depleting anti-Thy1.1 antibody several days before the infection. In this group transferred Tregs were present only during immune reconstitution, but not the infection. Another group of mice was adoptively transferred Tregs only after immune reconstitution was complete so that Tregs would be absent during LIP, but present during the infection (Fig. 3-4A).

We found that the total numbers of responder T cells in the lymphoid tissues after LIP and *L. monocytogenes* infection were identical. However, the percentages and absolute numbers (Fig. 3-5) of IA^b-2W1S-specific CD4 T cells and K^b-OVA-specific CD8 T cells, enumerated using magnetic enrichment and flow cytometry, revealed that presence of Tregs during LIP significantly enhanced expansion of antigen-specific T cells. In fact, the percentage of antigen-specific T cells in reconstituted RAG-/- mice that had Tregs present during LIP was similar to those seen in wild-type animals, although the absolute numbers were somewhat lower reflective of the lower total T cell

populations in reconstituted RAG-/- animals. Depletion of Tregs by anti-Thy1.1 mAb after T cell reconstitution was completed did not impact expansion of *Listeria*-specific responder T cells during the infection. In contrast, RAG-/- mice allowed to reconstitute in absence of Tregs had very few antigen-specific T cells, and there was no significant difference seen between animals that never received Tregs or received Tregs after LIP was already completed. Virtually all measured pathogen-specific T cells derived from the naïve responder T cell population and not the Thy1.1+ Tregs. This observation is consistent with the results reported by Ertelt et al. where infection by the same attenuated strain of *Listeria* used in our experiments was not seen to cause expansion of *Listeria*-specific Foxp3+ T cells.

Presence of Tregs during immune reconstitution preserves functional responsiveness of the T cell population.

Production of interferon-g by adaptive and innate immune systems contributes significantly to clearance of intracellular pathogens such as *L. monocytogenes*. Therefore, it would be reasonable to presume that numbers of *Listeria*-specific CD4 T cells that can produce IFN-γ relate to the immune fitness of an individual animal challenged with this infection. In order to examine this question, we repeated some experiments described above using TCRα-/- recipients (Supplemental Fig. 3-1). Once again, animals were reconstituted with conventional naïve T cells in the presence or absence of Tregs. Some animals that received Tregs before T cell reconstitution were treated with the depleting antibody following completion of LIP. Some animals that did not have Tregs during the period of LIP, were adoptively transferred with Tregs

following LIP completion. IFN-γ production was stimulated in vivo by intravenous injection of 5 x 10^7 CFU of live ActA$^-$ LM-2W1S-OVA 2-3 hours prior to harvest of the lymphoid tissues. We found that if Tregs were present during LIP, but not after, the reconstituted animals had more antigen-specific CD4 T cells overall, and more antigen-specific CD4 T cells capable of producing IFN-γ.

Finally, we tested whether *Listeria*-specific effector T cells generated within T cell populations that emerged in the course of immune reconstitution in the presence or absence of Tregs were capable of expansion when re-challenged with the pathogen (Supplemental Fig. 3-4). RAG-/- mice were repopulated with naïve Thy1.1 T cells in the presence or absence of Thy1.2 Tregs. After 30 days of reconstitution all animals were infected with 10^7 ActA$^-$ LM-2W1S-OVA bacteria. 10 days after infection, which is the peak of expansion by antigen-specific T cells in this system, lymphoid tissues of the animals were harvested and T cells that derived from the conventional T cell inoculum (Thy1.2$^-$ T cells in this experiment) were purified by negative selection. New wild-type recipients received 3 x 10^6 of these purified T cells, and were challenged with 10^7 ActA$^-$ LM-2W1S-OVA bacteria. Some wild-type recipients did not receive T cells from reconstituted RAG-/- animals and were used as controls. All mice were sacrificed on day 5 after the infection and lymphoid tissues were harvested for analysis. The results were dramatic. If Tregs were present during LIP, we could detect significant numbers of Thy1.1 CD4 T cells, most of which were specific for the 2W1S epitope. If Tregs were absent during LIP the numbers of 2W1S-specific CD4 T cells were indistinguishable from the numbers in the pre-immune repertoire. Similar general pattern was seen for the K^b-OVA-specific CD8 T cells.

Discussion

Discovery and characterization of CD25+Foxp3+ T cells in recent years has resurrected the concept of suppressor T cells playing a critical role in the maintenance of immunologic tolerance. These cells were termed 'regulatory' to be more encompassing of their potential functions, although they were shown to be truly suppressive to virtually every immunologic response studied. Multiple reasons can be cited to explain the abandonment of the suppressor T cell paradigm in 1980s, but one of them is its apparent conceptual inferiority in comparison to *antigen-specific* recessive mechanisms of tolerance such as clonal anergy and deletion. Today the existence of regulatory/suppressor T cells is no longer in question since we have physical markers that identify them, and although our mechanistic understanding for their suppressive activity remain incompletely understood, numerous potential mechanisms have been described. Nevertheless, the same theoretical question can be raised – why are suppressor T cells needed in our immune system?

The idea of suppressor T cells may seem more appealing if their antigen specificity compliments the antigen specificity of purely recessive mechanisms of tolerance. In fact, the repertoire of Tregs appears to be at least as diverse as that of conventional T cells, but it is also largely different. Most evidence suggests that the TCRs of thymic-derived Tregs have higher affinities for self-antigens than conventional T cells (55). Therefore, one could speculate that since Tregs need to be activated by their TCR to be functional, they may have the capacity to preferentially dampen immune responses in the settings where greater amounts of self-antigen are being

presented. Certainly, there is a great deal of clinical interest in antigen-specific Treg induction because antigen specificity can increase their suppressive potency by targeting their activity.

The function of suppressor T cells in infectious disease may be more nuanced. Tregs can inhibit or delay pathogen clearance, which at first glance may seem detrimental to the host. However, in some models, such as leishmaniasis and schistosomiasis, antigen persistence ensured by Tregs contributes to maintenance of immunity to reinfection. In other models, such as HSV-1 infection, Tregs were shown to inhibit immunopathology caused by inflammatory response that would otherwise severely disable the host (170). These are some examples that illustrate inadequacy of a simplistic view of the immune response, where stronger reaction is assumed to be better. In fact, in these cases of chronic infections Tregs may prevent winning a 'battle' with a pathogen, but at the same time help to win the 'war'. Of course, the paradigm of 'war' does not apply well to most of the activity of the immune system. The majority of the immune effort is concentrated at the mucosal surfaces, where it helps to maintain homeostasis with the commensal microbial flora and aid the physical barrier to exclude pathogen entry. That is one of the main functions of the secreted IgA, for example, which requires transforming growth factor-β (TGF-β) for class switching, one of the cytokines elaborated by Tregs (171).

At the outset we were intrigued by the differential ability of Tregs to inhibit spontaneous versus homeostatic forms of LIP in our experiments. This finding suggested that Tregs could play another positive role in the function of the immune system. Specifically, we thought they might optimize the immune fitness of the

residual T cell population during its recovery from lymphopenia by shaping its TCR repertoire. Lymphopenia is a common occurrence during lifetimes of individuals and peripheral mechanisms of immune reconstitution assume increasing importance with age as the thymus involutes. Our experimental adoptive transfer system models absence of the thymus and allows recovery of the T cell population exclusively by LIP.

We specifically wanted to start out with only minimally restricted potential TCR repertoire in order to both mimic realistic clinical scenarios, e.g., recovery of the T cell population in AIDS patients treated with highly active anti-retroviral therapy (HAART), and provide an experimental window to measure significant differences (172). A normal mouse contains about 2 x 10^6 T cell clones within its naïve TCR repertoire, and a starting population significantly below that would ensure severely restricted responsiveness to potential foreign antigens. In contrast, a starting population significant above that would limit LIP because there would be little lymphopenia. We employed p-MHC tetramers to probe the responsiveness of T cell populations to two foreign peptides, OVA and 2W1S, expressed within an infectious pathogen, *L. monocytogenes*. Enumeration of K^b-OVA- and IA^b-2W1S-specific T cells in the naïve repertoire reported previously and reproduced in our own work suggested that adoptive transfer of 1 x 10^6 T cells would result in transfer of about 2-4 T cells per mouse specific for these antigens. In fact, it has been shown that a single precursor T cells is capable of massive clonal expansion and functional subset diversification following immunization (173). Therefore, we expected to detect significant antigen-specific T cell expansion in all our experimental conditions in absence of significant distortion of the T cell repertoire during the period of immune reconstitution.

We found that the greatest numbers of responder foreign antigen-specific T cells recovered after infection were seen only in mice that had Tregs during the period of LIP. The simplest explanation for this finding is that some antigen-specific precursors from the naïve T cell inoculum did not survive the immune reconstitution period in absence of Tregs. In fact, in some experiments when Tregs were absent during LIP we couldn't detect any antigen-specific responder T cells at all following infection, and in others they were markedly decreased in numbers. This idea is also supported by the results of characterization of the TCR repertoire obtained by TCR Vβ-Jβ spectratyping, which demonstrated that absence of Tregs during immune reconstitution correlated with development of marked loss of structural TCR diversity. One would predict that the diversity of the few recovered antigen-specific responder T cells from populations established in the absence of Tregs would be extremely constricted and likely monoclonal. In fact, we have done several experiments using flow cytometric analysis Vβ chain usage by antigen-specific responders, and these were compatible with that prediction. Specifically, the Vβ chain usage of antigen-specific 2W1S-specific responders in mice reconstituted in presence of Tregs was comparable to the repertoire of 2W1S-specific responders in wild-type mice. The Vβ chain repertoire of 2W1S-specific responders in mice reconstituted in absence of Tregs appeared distorted. However, because of the small numbers of antigen-specific T cells in these mice and their complete absence in some experiments, the total number of mice necessary to characterize the Vβ usage by these cells was prohibitive. It should be interesting to focus future studies on the characteristics of the residual T cells that persist within the T cell population established by LIP in absence of Tregs. We might anticipate, for

example, that these cells have greater affinity for self-antigens that provide them with more tonic survival signals. Such cells might not necessarily have highest affinity for foreign antigens that the organism may encounter.

Our results indicate that the beneficial role of Tregs on the antigen-specific T cell response is exerted during the period of LIP (Fig. 3-6). Tregs adoptively transferred after completion of immune reconstitution had no beneficial effect. Furthermore, if Tregs were depleted following immune reconstitution, the beneficial effect persisted. It should be noted that Tregs were depleted using an antibody directed against congenic marker, Thy1.1. While that was very effective in depleting Thy1.1+ T cells, early presence of Tregs also appeared to encouraged some emergence of Foxp3+ T cells derived from the naive responder T cell population (Table 3-1). This might represent an example of 'infectious tolerance', and is consistent with the idea that factors produced by Tregs are somehow needed for Treg induction. However, the effect was relatively small and the numbers of induced Tregs in this group was considerably smaller than that produced by late adoptive transfer of Tregs. In both cases Tregs appearing after completion of LIP did not enhance the diversity of the reconstituted responder T cell population, and thereby its immune fitness.

The mechanisms of how Tregs may shape the TCR repertoire during LIP remain unclear at this time. At least several attractive models seem to be reasonable and non-mutually exclusive candidates. We started these studies after documenting that Tregs selectively inhibit the spontaneous form of LIP. Spontaneous LIP likely results from cognate antigen stimulation because it is inhibited by intraclonal competition and requires co-stimulation by B7:CD28 signaling. In contrast, co-stimulatory signals are

not needed for the homeostatic form of LIP, which is in turn driven mainly by mere tonic TCR signals and cytokines such as IL-7. It is reasonable to speculate that T cells undergoing both forms of LIP do utilize some common resources, e.g., IL-7, which may not be used significantly by Tregs. At the same time, Tregs and T cells undergoing spontaneous LIP may compete for another common resource, e.g., IL-2, which is known to be important for both Tregs and memory CD8 T cells. In this situation, suppression of spontaneous LIP by Tregs will liberate more resources for the rest of the T cells and prevent their extinction during immune reconstitution. Alternatively, Tregs may somehow selectively identify T cells undergoing spontaneous LIP and suppress them by blocking their cell cycle progression or inducing their apoptosis. Once again, more resources will be made available the majority of T cells that are not undergoing spontaneous LIP. Investigation of mechanism awaits future studies, and these phenomena may be in play in emerging clinical protocols. Thus, T cell depletion is commonly used in solid organ transplantation and we anticipate that costimulatory B7 blockade used in that setting may have protective effects on the T cell repertoire during recovery from lymphopenia. In addition, therapeutic adoptive transfer of Tregs is being developed for clinical use in organ transplantation, and their effects on immune fitness would be interesting and important to measure.

FIGURE 3-1. Filling of the T cell niche in RAG$^{-/-}$ mice through adoptive transfer. A titration of CD44^{lo} T cell numbers were transferred into C57BL/6 RAG-1$^{-/-}$ recipients on Day 0 (two recipients per condition). Major lymph nodes (cervical, brachial, axillary, inguinal, and mesenteric) and spleens were harvested on Days 7 and 30, stained for T cell markers (CD4 and CD8) and a panel of antibodies reactive to 14 known mouse TCR Vβ chains, and analyzed by FACS.

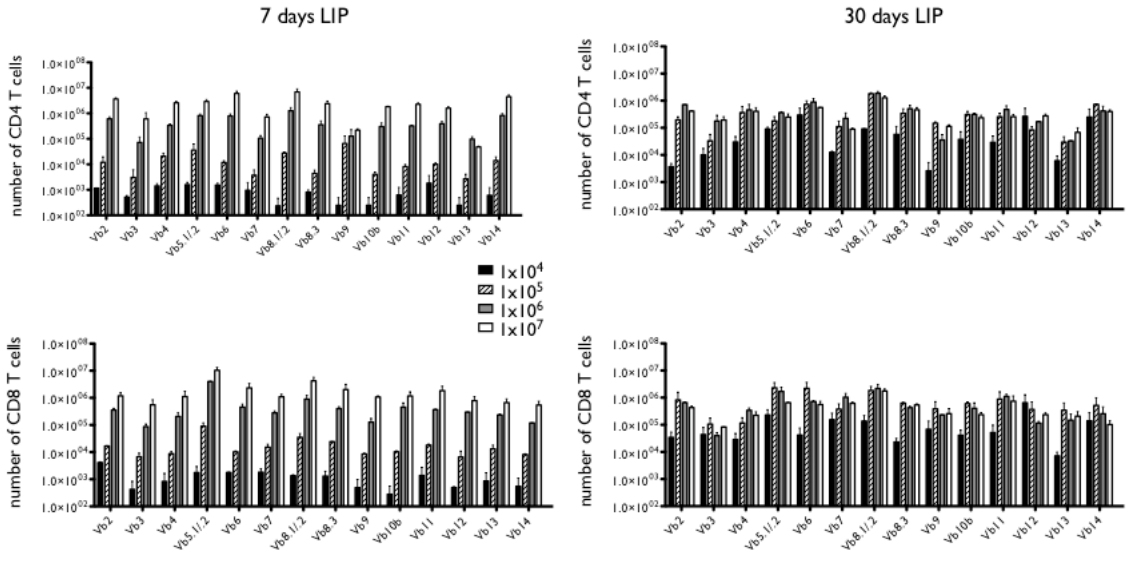


FIGURE 3-2. Assessing T cell diversity post-LIP at the level of TCR Vβ chain usage. *A*. The experiment protocol for assessment of *structural* T cell diversity post-LIP in the presence or absence of Tregs involves two separate measurements from each sample: TCR Vβ surface staining for FACS, and RNA isolation and spectratyping. *B*. Animals were sacrificed and lymphoid tissue samples were processed on Day 30. Half of each processed sample was treated to cell surface staining with antibodies specific to T cell markers (CD4, CD8, Thy1.2) and FACS analysis. *C*. The remaining half of each sample was processed for RNA and TCR Vβ spectratyping. Each graphed data bar or point represents an average of 3-4 animals total from 2 independent experiments. Complexity scores were calculated from spectratyping of Vβ 10 and 8.1 (experiment #1), and from Vβ 10 and 8.3 (experiment #2).

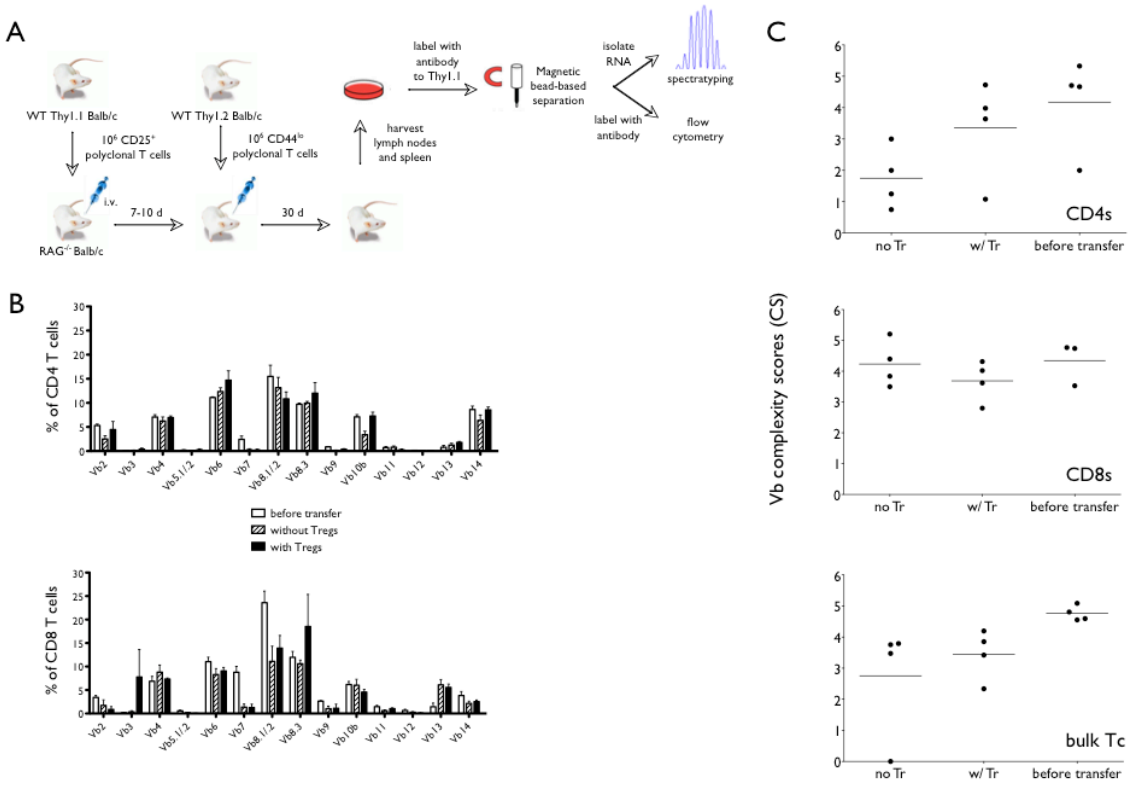


FIGURE 3-3. Tregs prevent emergence of 'holes' in the T cell repertoire during LIP. *A*. Samples from the experiments described in Fig. 3-2 PCR amplified for Vβ10 expression were spectratyped for CDR3 diversity by further PCR amplification for Jβ1.5. Each bar graph depicts a single, representative animal sample. *B*. An average complexity score for each Jβ chain was calculated based on spectratyping samples from experiments described above. A mean value representing data from all 12 chains under each treatment condition (LIP +/- Tregs) was used to determine statistical significance from before transfer samples. All conditions were significant with respect to 'before transfer' scores.

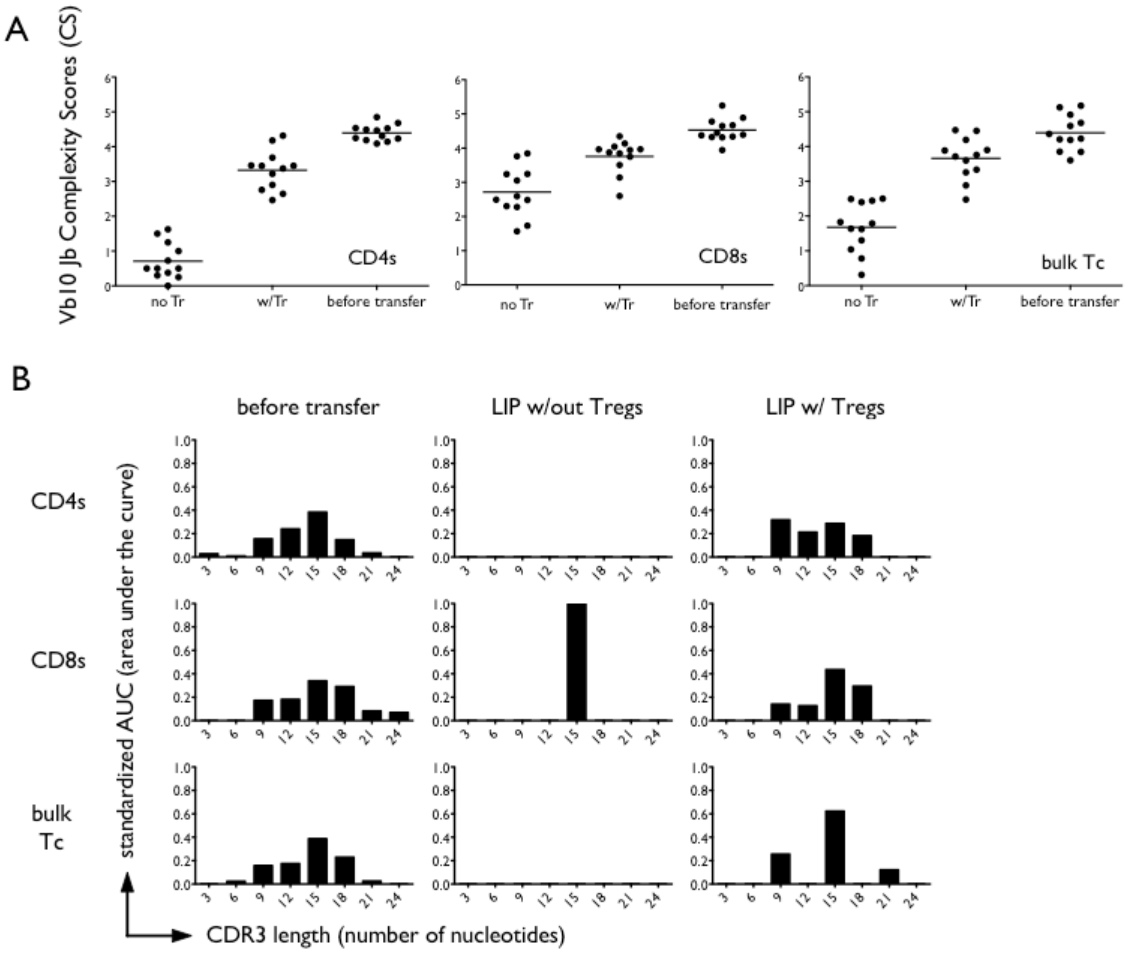
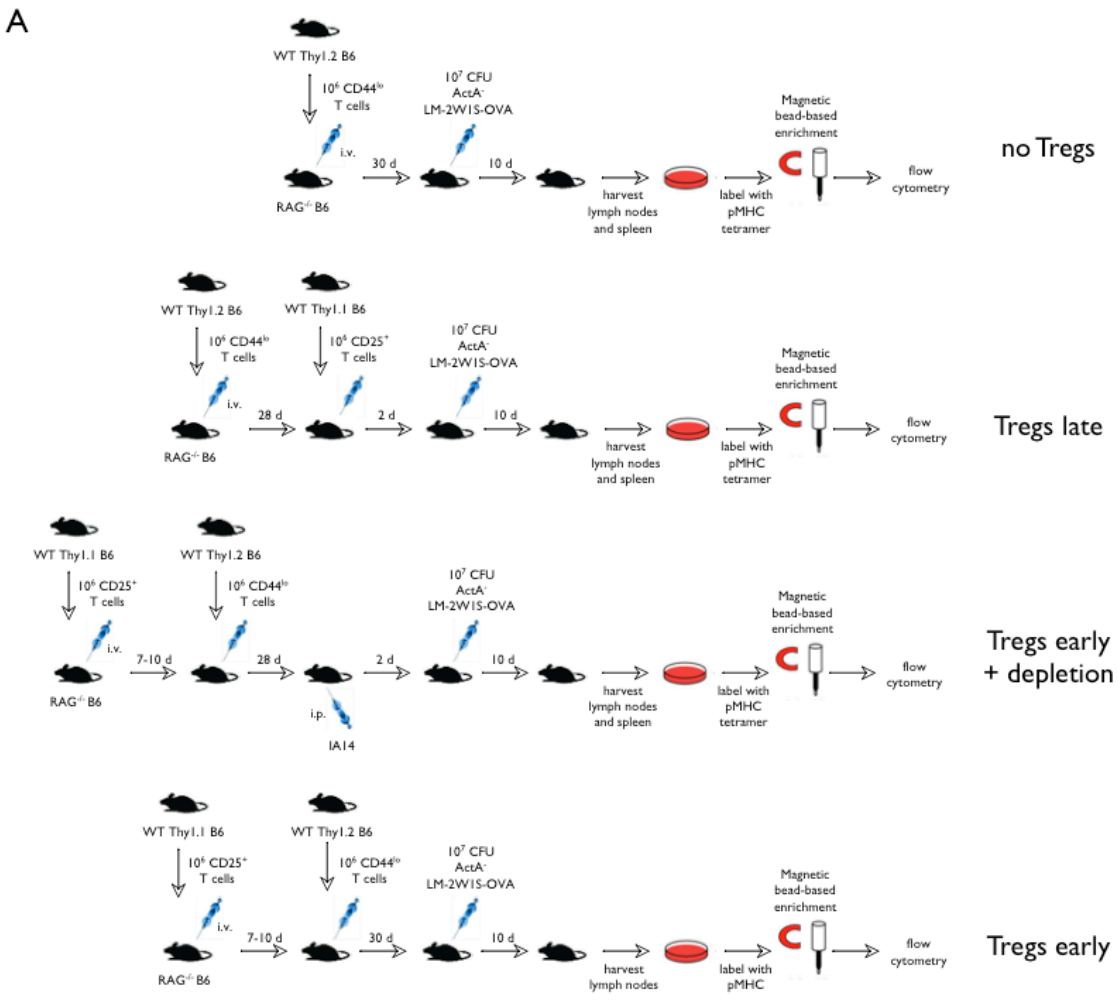
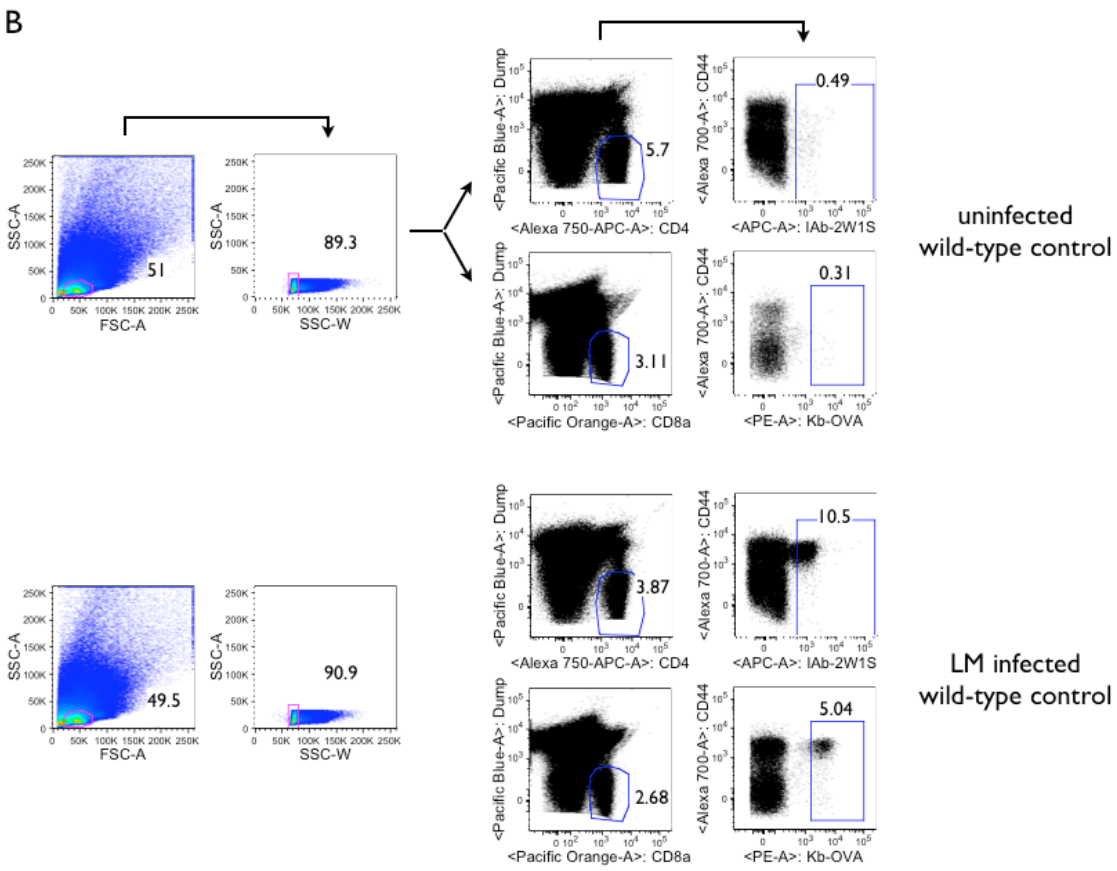


FIGURE 3-4. Tregs preserve the diversity of foreign Ag-reactive T cells during LIP. *A*. The basic experiment protocol for assessment of *functional* diversity post-LIP in the presence or absence of Tregs involves lymphoid tissue harvest and tetramer based-enrichment for Ag-specific cells post-bacterial infection. *B*. Plots depict the gating strategy using the control group samples from one representative experiment in which two animals per group were processed, enriched, acquired by FACS, and analyzed together. *C*. Depicted are samples from one representative experiment. Numbers represent percent gated of total population within each plot. Tr late = Tregs added after LIP, but before infection (Day 28); Tregs were depleted from the third group of animals on Day 28.


A
WT Thy1.2 B6
10^6 CD44^lo T cells
i.v.
RAG^-/- B6
30 d
10^7 CFU ActA- LM-2W1S-OVA
10 d
harvest lymph nodes and spleen
label with pMHC tetramer
Magnetic bead-based enrichment
flow cytometry
no Tregs
WT Thy1.2 B6
WT Thy1.1 B6
10^6 CD44^lo T cells
10^6 CD25+ T cells
i.v.
RAG^-/- B6
28 d
2 d
10^7 CFU ActA- LM-2W1S-OVA
10 d
harvest lymph nodes and spleen
label with pMHC tetramer
Magnetic bead-based enrichment
flow cytometry
Tregs late
WT Thy1.1 B6
WT Thy1.2 B6
10^6 CD25+ T cells
10^6 CD44^lo T cells
i.v.
RAG^-/- B6
7-10 d
28 d
i.p.
IA14
2 d
10^7 CFU ActA- LM-2W1S-OVA
10 d
harvest lymph nodes and spleen
label with pMHC tetramer
Magnetic bead-based enrichment
flow cytometry
Tregs early + depletion
WT Thy1.1 B6
WT Thy1.2 B6
10^6 CD25+ T cells
10^6 CD44^lo T cells
i.v.
RAG^-/- B6
7-10 d
30 d
10^7 CFU ActA- LM-2W1S-OVA
10 d
harvest lymph nodes
label with pMHC
Magnetic bead-based enrichment
flow cytometry
Tregs early


B
SSC-A
FSC-A
51
SSC-W
89.3
<Pacific Blue-A>: Dump
<Alexa 750-APC-A>: CD4
5.7
<Alexa 700-A>: CD44
<APC-A>: IAb-2W1S
0.49
<Pacific Orange-A>: CD8a
3.11
<PE-A>: Kb-OVA
0.31
uninfected
wild-type control
49.5
90.9
3.87
10.5
2.68
5.04
LM infected
wild-type control

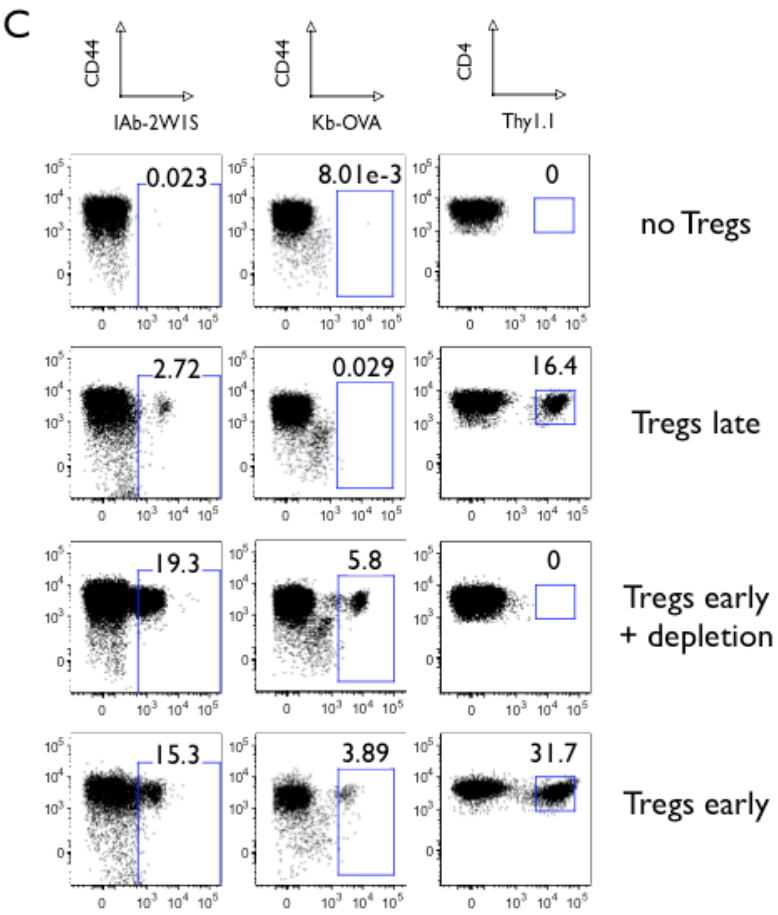

C
CD44
IAb-2W1S
CD44
Kb-OVA
CD4
Thy1.1
0.023
8.01e-3
0
no Tregs
2.72
0.029
16.4
Tregs late
19.3
5.8
0
Tregs early
+ depletion
15.3
3.89
31.7
Tregs early

FIGURE 3-5. The presence of Tregs during LIP ensures responsiveness of foreign Ag-reactive T cells to bacterial infection. Data represent total and tetramer-enriched T cells from six independent experiments of 1 to 2 animals per group. Note: no K^b-OVA – specific events were found in LIP hosts from one of six experiments.

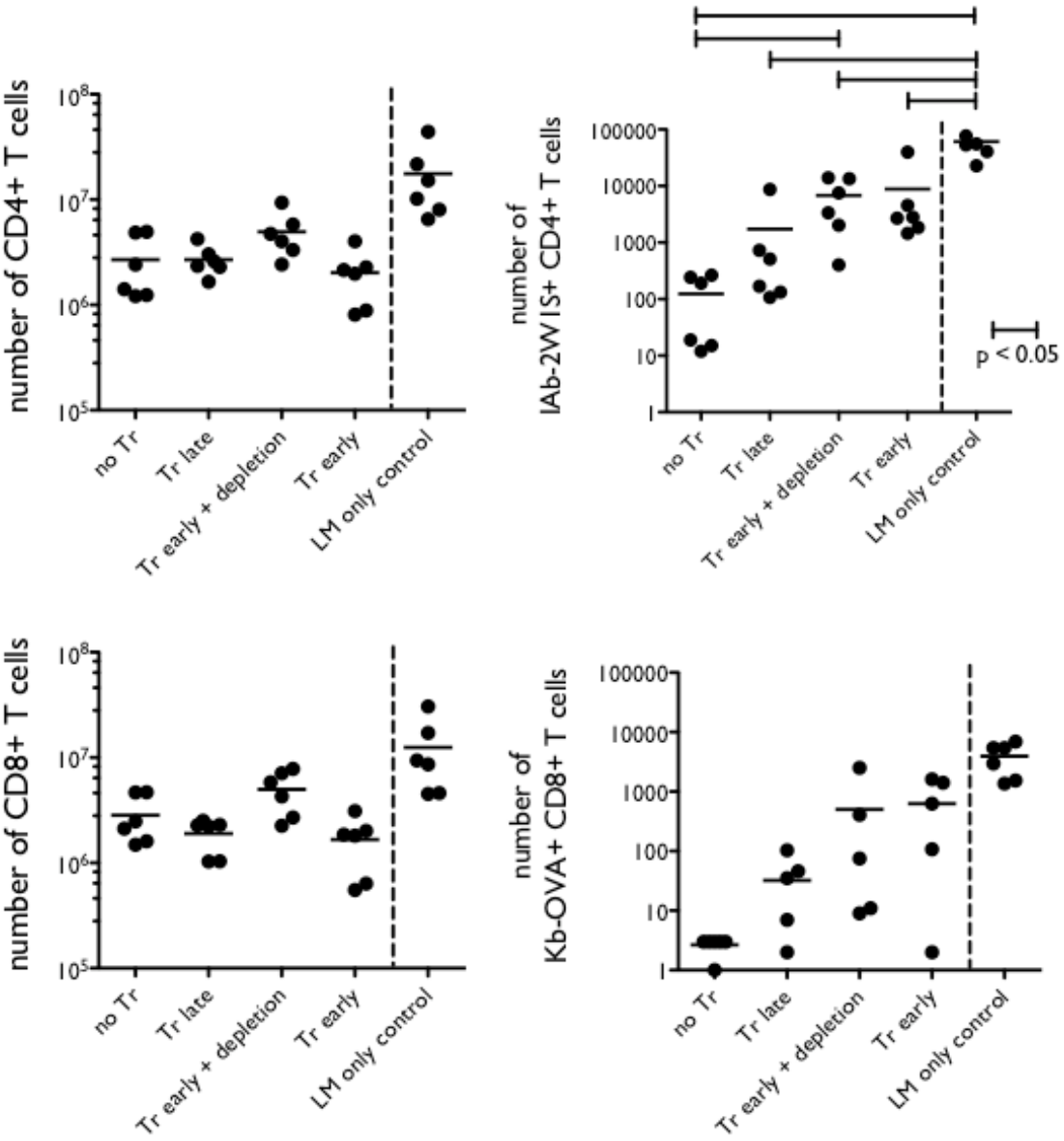
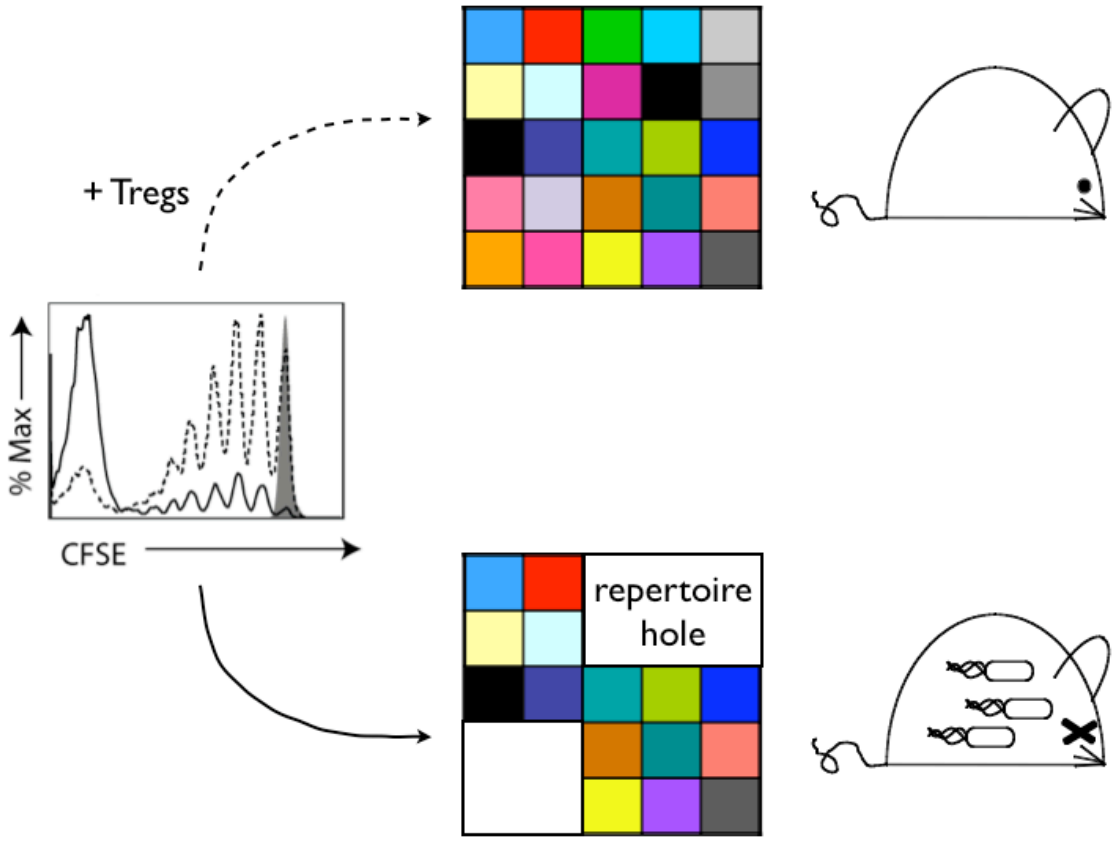


TABLE 3-1. LIP and subsequent bacterial infection do not induce significant peripheral conversion of conventional T cells to Foxp3+. Data represents mean +/- standard deviation of two independent experiments of two animals per group, conducted as described in Fig. 3-4.

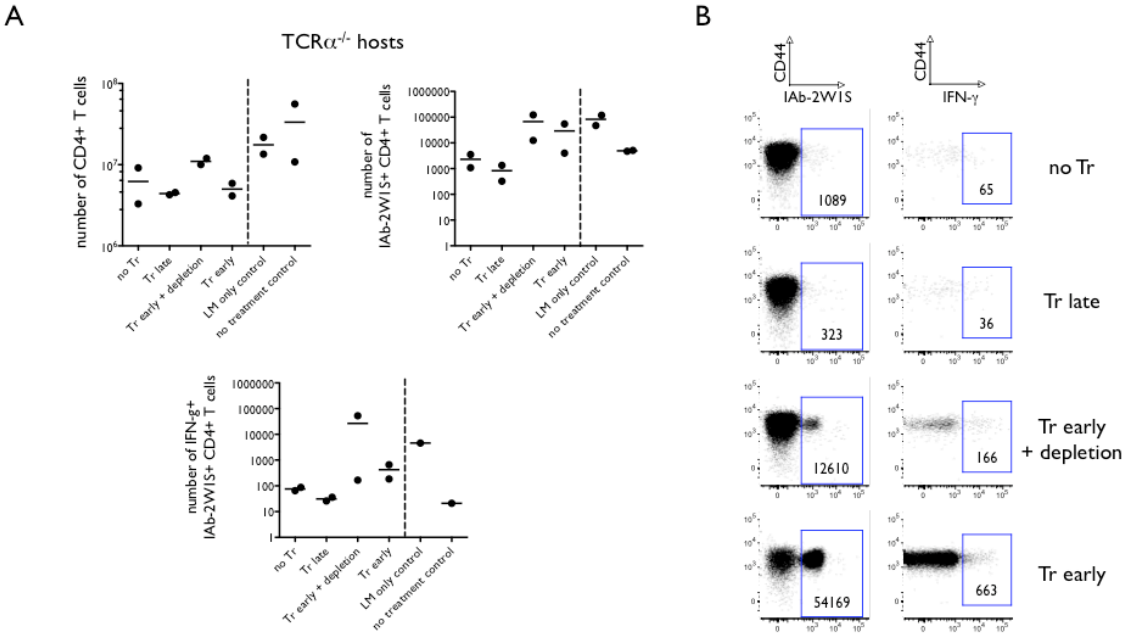
LIP sample	% Thy1.1+ that are Foxp3+	Thy1.1+Foxp3+ T cell number	% Thy1.2+ that are Foxp3+	Thy1.2+Foxp3+ T cell number	% Foxp3+ of total CD4 T cells
Tr early	56.4 +/- 6.0	247526 +/- 106369	1.9 +/- 0.1	19666 +/- 13161	18.5 +/- 3.0
Tr late	61.5 +/- 9.6	93409 +/- 37418	0.7 +/- 0.4	12463 +/- 2280	4.6 +/- 0.4
no Tr			1.6 +/- 0.1	50595 +/- 43626	1.6 +/- 0.1
Tr early + depletion	25.5 +/- 4.4	358 +/- 325	5.3 +/- 2.8	247743 +/- 239088	5.2 +/- 2.8

wild-type sample	% Foxp3+	Foxp3+ T cell number
LM only	6.9 +/- 2.5	637252 +/- 329660
no Tx	10.0 +/- 4.0	1357831 +/- 939317

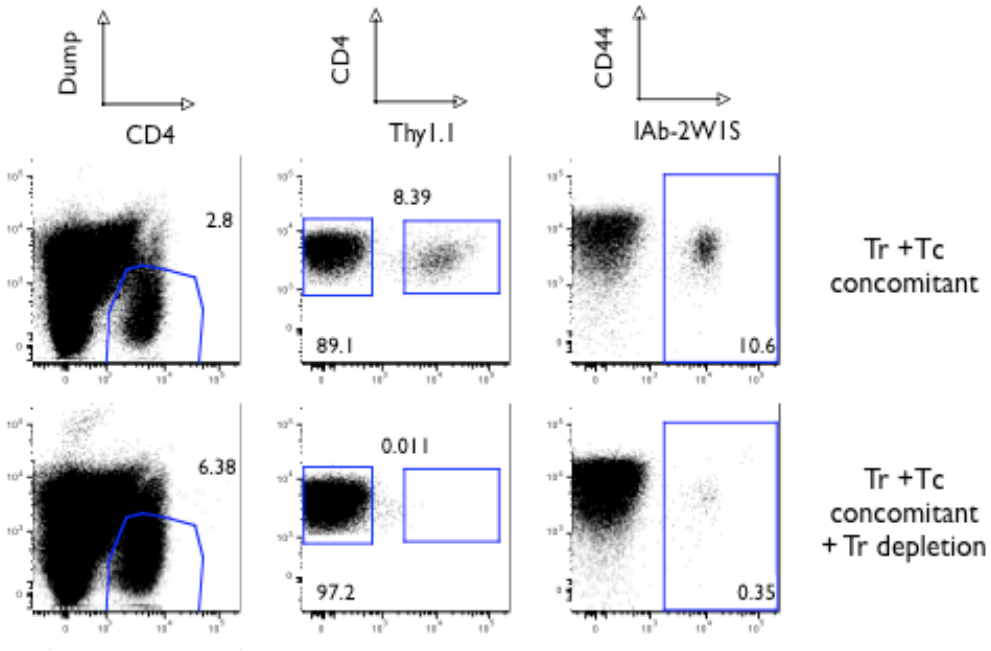
FIGURE 3-6. Tregs preserve immune fitness of conventional T cells undergoing LIP. The CFSE profile depicted on the left side of this model figure illustrates control of T cell LIP by Tregs within the secondary lymphoid tissues. The presence of Tregs limits spontaneous LIP, which in turn limits the potential for holes in the repertoire to develop and preserves diversity upon reconstitution (colored squares). As depicted on the right side of the figure, the outcome of preserved T cell repertoire fitness is invading pathogen clearance and/or suppression of damaging inflammation and breach of tolerance to commensals.



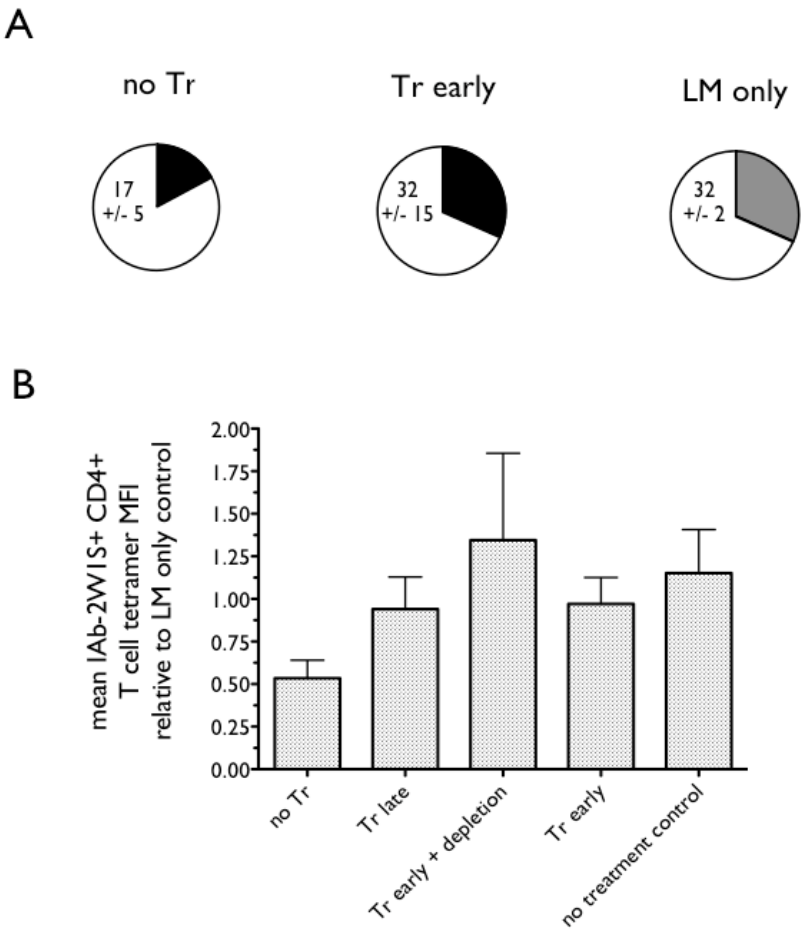
SUPPLEMENTAL FIGURE 3-1. Tregs preserve the diversity of foreign Ag-reactive T cells during LIP in TCR$\alpha^{-/-}$ hosts. LIP experiments were conducted as described in Fig. 3-4, with two exceptions. First, TCR$\alpha^{-/-}$ hosts were used in place of RAG$^{-/-}$ hosts for LIP. Second, host mice were challenged with LM as described in the Materials and Methods section for the purpose of intracellular staining for IFN-γ and assessment of functional Ag-specific T cell fitness. *A*. Each data point in the plots represents the average of 2 animals per group from 2 independent experiments (4 animals total per condition). *B*. Each set of 2 FACS plots reflects a representative sample from each group (tissue from 2 animals processed, stained, and acquired together). Numbers represent percent gated of total population within each plot.



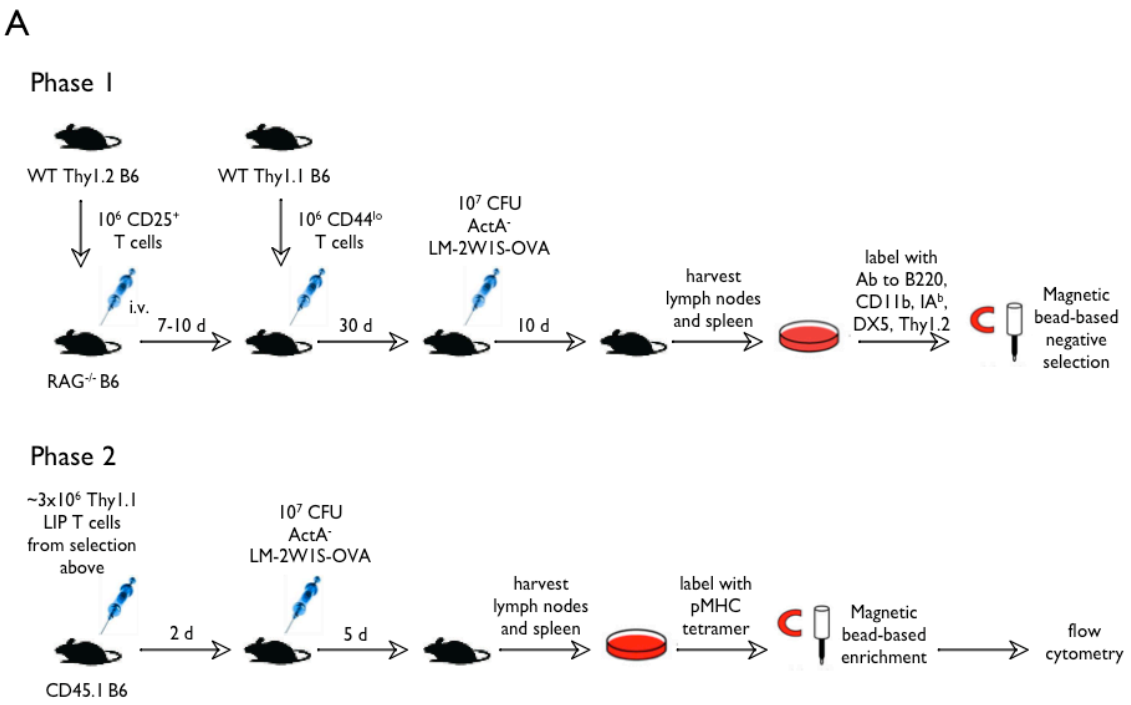
SUPPLEMENTAL FIGURE 3-2. Tregs concomitantly transferred with conventional T cells preserve the diversity of conventional CD4 T cell foreign Ag-reactivity during LIP. Depicted are samples from a single experiment conducted as in Fig. 3-4. Numbers represent percent gated of total population within each plot. Highlighted with a black box are groups corresponding to concomitant transfers of $1x10^6$ Treg and $1x10^6$ Tconv on day 0.



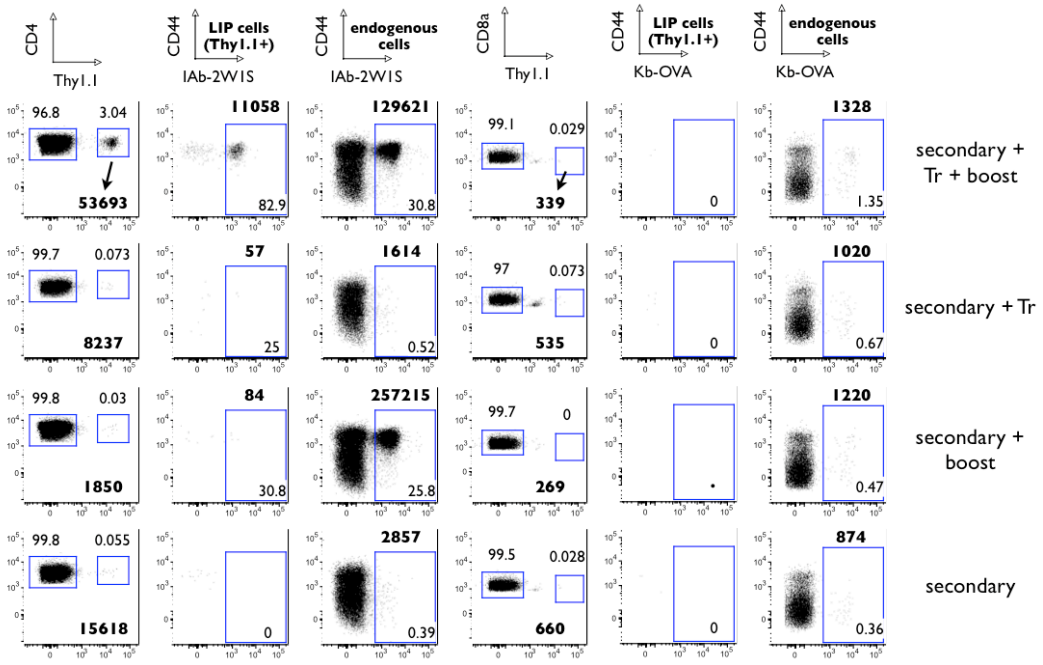
SUPPLEMENTAL FIGURE 3-3. Tregs preserve T cell diversity during LIP as assessed by correlative measures. Data reflects staining of IA^b-2W1S tetramer$^+$ cells with a panel of TCR Vβ 4, 5, 11 antibodies (*A*) and measures of mean fluorescence intensity of tetramer$^+$ cells gated as depicted in Fig. 3-4 (*B*). Numbers within pie charts depict mean +/- SEM of 4-6 individual experiments of 2 animals/experiment/condition.



SUPPLEMENTAL FIGURE 3-4. Isolated and re-transferred LIP cells respond to challenge in a secondary, lymphoreplete host. *A*. A single experiment of two animals per group was conducted as outlined in Fig. 3-4A (Phase 1) with the following exceptions: lymph node and spleens were harvested post-LIP in presence or absence of Tregs, followed by conventional T isolation and retransfer (3 x 10^6 each) into secondary, lymphoreplete hosts for LM challenge (Phase 2). *B*. Bolded numbers in plots represent absolute number of gated cells. Regular (non-bolded) numbers represent percent gated of total cells within each plot.



B


CD4
Thy1.1
CD44
LIP cells (Thy1.1+)
IAb-2W1S
CD44
endogenous cells
IAb-2W1S
CD8a
Thy1.1
CD44
LIP cells (Thy1.1+)
Kb-OVA
CD44
endogenous cells
Kb-OVA
96.8
3.04
53693
11058
82.9
129621
30.8
99.1
0.029
339
0
1328
1.35
secondary + Tr + boost
99.7
0.073
8237
57
25
1614
0.52
97
0.073
535
0
1020
0.67
secondary + Tr
99.8
0.03
1850
84
30.8
257215
25.8
99.7
0
269
•
1220
0.47
secondary + boost
99.8
0.055
15618
0
2857
0.39
99.5
0.028
660
0
874
0.36
secondary

SUPPLEMENTAL TABLE 3-1. Primers used for TCR spectratyping (5' → 3').

Cbeta 1A	GTT TGA GCC ATC AAA AGC AGA
Cbeta 3C	AGG ATC TCA TAG AGG ATG GT
Beta actin frwrd	GTG GGC CGC TCT AGG CAC CAA
Beta actin bckwrd	CTC TTT GAT GTC ACG CAC GAT TTC
C beta	CAC TGA TGT TCT GTG TGA CA
	6-FAM/CAC TGA TGT TCT GTG TGA CA
V beta 1	CTG AAT GCC CAG ACA GCT CCA AGC
V beta 2	TCA CTG ATA CGG AGC TGA GGC
V beta 3.1	CCT TGC AGC CTA GAA ATT CAG T
V beta 4	GCC TCA AGT CGC TTC CAA CCT C
V beta 5.1	CAT TAT RGA TAA AAT GGA GAG AGA T
V beta 5.2	AAG GTG GAG AGA GAC AAA GGA TTC
V beta 5.3	AGA AAG GAA ACC TGC CTG GTT
V beta 6	CTC TCA CTG TGA CAT CTG CCC
V beta 7	TAC AGG GTC TCA CGG AAG AAG C
V beta 8.1	CAT TAC TCA TAT GTC GCT GAC
V beta 8.2	CAT TAT TCA TAT GGT GCT GGC
V beta 8.3	TGC TGG CAA CCT TCG AAT AGG A
V beta 9	TCT CTC TAC ATT GGC TCT GCA GGC
V beta 10	ATC AAG TCT GTA GAG CCG GAG GA
V beta 11	GCA CTC AAC TCT GAA GAT CCA GAG C
V beta 12	GAT GGT GGG GCT TTC AAG GAT C
V beta 13	AGG CCT AAA GGA ACT AAC TCC CAC
V beta 14	ACG ACC AAT TCA TCC TAA GCA C
V beta 15	CCC ATC AGT CAT CCC AAC TTA TCC
V beta 16	CAC TCT GAA AAT CCA ACC CAC
V beta 17	AGT GTT CCT CGA ACT CAC AG
V beta 18	CAG CCG GCC AAA CCT AAC ATT CTC
V beta 19	CTG CTA AGA AAC CAT GTA CCA
V beta 20	TCT GCA GCC TGG GAA TCA GAA
J beta 1.1	6-FAM/ACT GTG AGT CTG GTT CCT TTA CC
J beta 1.2	6-FAM/AAA GCC TGG TCC CTG AGC CGA AG
J beta 1.3	6-FAM/CTT CCT TCT CCA AAA TAG AGC
J beta 1.4	6-FAM/GAC AGC TTG GTT CCA TGA CCG
J beta 1.5	6-FAM/GAG TCC CCT CTC CAA AAA GCG
J beta 1.6	6-FAM/TCA CAG TGA GCC GGG TGC CTG C
J beta 2.1	6-FAM/GTG AGT CGT GTT CCT GGT CCG AAG
J beta 2.2	6-FAM/CCA GCA CTG TCA GCT TTG AGC
J beta 2.3	6-FAM/GTT CCT GAG CCA AAA TAC AGC G
J beta 2.4	6-FAM/GTG CCC GCA CCA AAG TAC AAG
J beta 2.5	6-FAM/GTG CCT GGC CCA AAG TAC TGG
J beta 2.7	6-FAM/CTA AAA CCG TGA GCC TGG TGC

Chapter 4

Regulatory CD4+CD25+Foxp3+ T cell suppression of conventional T cell LIP: potential mechanisms

Abstract

As discussed briefly in the first chapter of this thesis, the study of Treg suppressive mechanisms is a rapidly evolving field. The focus of much of this study involves mechanisms with potential for exploitation in clinical therapy for autoimmunity and cancer. The following thesis chapter encompasses preliminary data from single pilot experiments focusing on two of these potential mechanisms: 1) outside-in signaling from CTLA-4 expressed on Tregs through B7 expressed on IDO-expressing DC, and 2) competition between Tregs and conventional T cells for the homeostatic cytokine IL-2. Using materials and techniques described in previous chapters, we compare and contrast the influence of these suppressive mechanisms on acute T cell LIP and the 'chronic' LIP-based, adoptive transfer colitis model, and suggest directions for future research.

Materials and Methods

Mice

C57BL6 (B6), BALB/c, and B6 CD45.1 congenic mice were purchased from the National Cancer Institute (Frederick, MD). Recombinase-deficient (RAG-2$^{-/-}$) BALB/c mice were purchased from Taconic Farms (Germantown, NY). DO11.10 CD28$^{-/-}$ mice were bred as described previously (126, 174). Recombinase-deficient (RAG-1$^{-/-}$), MyD88$^{-/-}$, B7(-1 and -2)$^{-/-}$, CD28$^{-/-}$, and Thy1.1 congenic mice on the B6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). CTLA-4$^{+/-}$ and IDO$^{-/-}$ B6 mice were generous gifts from Drs. J. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY) and A. Mellor (Medical College of Georgia, Augusta, GA). IL-

$2^{-/-}$ RAG-$2^{-/-}$, IL-$15^{-/-}$ RAG-$2^{-/-}$, and $Bim^{-/-}$ on the B6 background were generously provided by Drs. M. Farrar and K. Hogquist at the University of Minnesota. RAG-$2^{-/-}$ OT-I TCR transgenic (Tg) mice (122) were bred onto the IL-$2^{-/-}$ RAG-$2^{-/-}$ background. $IDO^{-/-}$, $MyD88^{-/-}$, and $B7^{-/-}$ mice were bred on the $RAG^{-/-}$ B6 and BALB/c backgrounds in our facility. All mice used were generally 4-20 weeks of age. All animals were maintained in a specific pathogen-free facility in microisolator cages with filtered air according to the National Institutes of Health guidelines.

Adoptive transfer and cell preparations

Unless otherwise specified, donor T cells were collected from secondary lymphoid tissues (axillary, brachial, cervical, mesenteric, and inguinal lymph nodes, and spleen). Polyclonal $CD44^{low}$ CD8 and CD4 T cells were purified in two stages—first, CD8 or CD4 T cells were prepared by negative selection against CD8 or CD4, MHC class II, CD11b, and B220 (all Abs labeled with FITC) using anti-FITC BioMag particles (Polysciences, Warrington, PA), and in some cases also anti-CD25-, GITR-, and CD103-FITC, followed by depletion of $CD44^{high}$ cells using magnetic microbeads (Miltenyi Biotec, Auburn, CA), as previously described (123). Briefly, the purified CD8 or CD4 T cells were suspended in labeling buffer (2% FCS in PBS), incubated with 0.004 μg anti-CD44-FITC (eBioscience, San Diego, CA) per 10^6 cells for 15 minutes, washed, and labeled with anti-FITC magnetic microbeads. The negative fraction was collected following Miltenyi Biotec magnetic column separation. OT-I RAG-$2^{-/-}$ TCR Tg CD8 T cells were either depleted for $CD44^{high}$ cells or used directly ex vivo / without purification from donor mice. $CD25^{+}$ T cells were prepared using

positive selection with anti-CD25 biotinylated mAb, PC61, and streptavidin-labeled magnetic microbeads (Miltenyi).

FACS analysis

T cells isolated for adoptive transfer were labeled with 7.5 μM CFSE (Molecular Probes, Eugene, OR) and i.v. injected into host mice. Mice were sacrificed with CO_2 for removal of spleen and lymph nodes. All secondary lymphoid tissues were disrupted by mashing with a syringe. Colons were digested in collagenase D (Roche Applied Science, Indianopolis, IN), 400 U ml^{-1} for 30 min at 37 °C. In some cases, the collagenase medium was supplemented with 10 μg ml^{-1} brefeldin A (Sigma, St. Louis, MO) to prevent lymphokine secretion. Colons were washed with PBS, cut into small segments and digested prior to staining for FACS. Intracellular staining for cytokines was done following stimulation with PMA/ionomycin and fixation. Specific T cell subsets were identified using fluorochrome-labeled antibodies against CD3e, CD4, CD8, and congenic markers such as anti-Thy1.1/Thy1.2 and anti-CD45.1/CD45.2. All anti-cytokine (IL-2, IL-17A, IFN-γ, and isotype controls) and anti-KLRG-1 antibodies were purchased from eBioscience (San Diego, CA). Granzyme B was stained intracellularly using anti-human granzyme B antibodies (Caltag Laboratories, Burlington, CA). Staining for Foxp3 was done using an eBioscience kit and instructions provided by the manufacturer. Absolute numbers of T cells within various tissues were calculated using PKH reference beads (Sigma-Aldrich, St. Louis, MO). Relative numbers of CD4 T cells of specific subtype were calculated in comparison to the number of total CD4 T cells in the group that did not receive Tregs.

Induction and assessment of colitis

Colitis was induced by i.v. adoptive transfer of 1 x 10^5 $CD4^+CD25^-CD103^-$ $CD44^{lo}GITR^{lo}$ cells purified using magnetic microbeads from wild-type donor mice, as described above, into $RAG^{-/-}$ animals. Some of the recipient mice were transferred $CD4^+CD25^+$ Tregs prior to the transfer of naïve CD4 T cells. The animals were monitored clinically by weights and signs of colitis and systemic toxicity. After sacrifice 1 cm distal-most part of the colon was removed and fixed in 10% buffered formalin. Parrafin-embedded sections were cut and stained with H & E. Severity of colitis was scored in a blinded fashion according to a scale similar to that previously described (121): grade 0, normal histologic appearance; grade 1, minimal scattered inflammatory cell infiltrates within the lamina propria, with or without minimal epithelial hyperplasia; grade 2, mild to moderate, scattered inflammatory infiltrates, sometimes extending into the submucosa, with mild to moderate epithelial hyperplasia and and mild to moderate depletion of mucin from goblet cells; grade 3, moderate inflammation in the lamina propria, sometimes transmural, occasional crypt abcesses, moderate to severe epithelial hyperplasia and mucin depletion; grade 4, severe inflammatory infiltration of lamina propria, transmural inflammation, abundant crypt abcesses, occasional ulceration, marked epithelial hyperplasia, and mucin depletion; grade 5, severe inflammation with diffuse loss of epithelium.

Direct cell-to-cell (e.g. CTLA-4 expression on Tregs; induction of IDO on APC; TLR recognition)

Regulatory T cells are known to express CTLA-4 constitutively, however, until very recently, the role this inhibitory signaling molecule played in Treg homeostasis and suppression of T cell responses was not well defined. Published reports suggest CTLA-4 expression is associated with Treg production of immunosuppressive cytokines TGF-β and IL-10 and is essential for cell-to-cell-suppression in vitro. Determining what role CTLA-4 expression on Tregs plays in vivo is complicated, however, due to differential expression on regulatory and non-regulatory T cells and development of fatal lymphoproliferative disease in CTLA-4-deficient mice. Basic research and clinical reports published in recent years suggest blockade of CTLA-4 on Tregs abrogates their in vivo function, allowing the generation of effective anti-viral and anti-tumor T cell responses and/or emergence of autoimmune disease (175-177).

Potential CTLA-4-mediated suppressive mechanisms include outside-in signaling through B7 and resultant upregulation/activation of tryptophan catabolism and suppression of T cell function/viability (65, 178). It is thought naturally occurring (thymus-derived) Tregs may function this way to prevent activation and expansion of potentially pathogenic T cell clones in response to infectious stimuli. Fallarino et al. speculate that suppression of an active immune response through activation of tryptophan catabolism by DCs and subsequent suppression of T cell effector function depends on immunosuppressive cytokines like IL-10 or IFN-γ that act in a paracrine or autocrine fashion. This may be salient to IL-10-dependent suppression of established

colitis by Tregs, assuming suppression is mediated primarily through tolerizing DC. Tregs are known to express TLRs and respond differentially depending on the ligand encountered (179-182). Fallarino and colleagues have published data to suggest TLR ligation on Tregs may induce production of IL-10 and/or IFN-γ, leading to activation of the enzyme indoleamine 2,3-dioxygenase (IDO) and induction of tryptophan catabolism in DCs with which they interact (178).

Results

Our working hypothesis regarding the function of CTLA-4 on Tregs was that outside-in signaling through B7 on APC and activation of IDO was essential to suppression of spontaneous T cell LIP. As a first step in testing our hypothesis, we took advantage of the fact that CD28-deficient T cells, known to undergo spontaneous LIP in a B7 and RAG double-deficient host, should not be influenced by any potential B7-independent co-stimulation (114) (Fig. 4-1A). Our expectation - that if Tregs are unable to ligate B7 on APC, they will be unable to suppress spontaneous proliferation – was supported for both naïve CD4 and CD8 T cells undergoing LIP (Fig. 4-1B). Importantly, the failure of Tregs to suppress naïve, conventional T cell spontaneous LIP was not a consequence of failure to undergo proliferation themselves in a B7-deficient host (Fig. 4-1C). Based on this encouraging preliminary data, we sought to determine a role for IDO expression directly using IDO-deficient mice we bred to a RAG-1-deficient background in our facility. Using adoptive transfer into these lymphopenic hosts, we first determined that host IDO expression was not requisite for spontaneous

LIP of naïve CD4 T cells and that Tregs themselves were not impaired for proliferation in these hosts (Fig. 4-2B – right panel). IDO expression by host cells in fact plays a part in dampening LIP, as evidenced by enrichment of transferred cells in double-deficient over RAG single-deficient hosts (Fig. 4-2B - left). However, contrary to expectation, Tregs pre-loaded into IDO-deficient hosts were able to suppress spontaneous LIP in the lymphoid tissue of subsequently transferred naïve, conventional CD4 T cells (Fig. 4-2A and B).

Given this perplexing result regarding IDO-independent Treg-mediated suppression of acute LIP in the lymphoid tissue, and considering the trend towards greater proliferation of naïve CD4 T cells transferred into an IDO-deficient, lymphopenic host, we were curious about Treg suppression in peripheral tissues. IDO expression is important for homeostasis of the maternal-fetal interface and is reportedly highly expressed in gut epithelia (183). We demonstrate in chapter 2 of this thesis that Treg-mediated suppression of spontaneous LIP is less than 100% complete. We also speculated that T cell clones undergoing LIP that escape Treg-mediated suppression is the basis for the requirement for IL-10-expressing Treg suppression in peripheral tissues. One would, therefore, expect IL-10 expression by Tregs to be essential for suppression of pathology in the LIP-based, adoptive transfer colitis model. Our initial experiment was consistent with this expectation, in that IDO-deficient recipients exhibited more severe colitis than WT recipients in this model (as evidenced by enrichment for IL-17 in MLNs and higher than WT colitis score – Fig. 4-3B, C, and D). However, we were surprised to find that IL-10-competent Tregs do not require IDO induction in the host as part of their mechanistic program for suppression (Fig. 4-3B).

As discussed above, Fallarino et al. speculate that Treg-secreted IL-10 acts directly on DC to induce tolerance and suppress potential pathogenicity. Furthermore, it is theoretically possible protection is manifested by *de novo* induction of Tregs (184, 185). As a basic experiment, IDO chimeras were generated to determine the source of IDO mediating suppression of colitis (hematopoietic or radio-resistant, non-hematopoietic host cells) and to determine whether conventional CD4 T cells transferred into these chimeras were converting into Tregs in the target tissues (MLN and colon) (Fig. 4-4A). Results of this experiment suggest deficiency in IDO expression in radio-resistant cells (likely intestinal epithelia) exacerbates developing colitis (Fig. 4-4B – KO as recipients of bone marrow), However, deficiency of IDO in hematopoietic, radio-sensitive cells actually appears to protect against inflammation early after transfer of conventional CD4 T cells. This necessity for IDO expression in recipient epithelia is consistent with conversion of a greater number of conventional CD4 T cells into a Foxp3+ phenotype in the colon (Fig. 4-4C).

We adopted the same experimental techniques to investigate the role of TLR signaling in Treg suppression of spontaneous LIP and LIP-induced colitis. We know from work published by Kieper et al. using germ-free animals that microflora play an important part in stimulating spontaneous proliferation of T cells in a lymphopenic host, and that presence of the same microflora are important for establishing tolerance during reconstitution (86). Based on this information, one could hypothesize expression of the myeloid differentiation primary response gene (88) (MyD88), the major adaptor molecule downstream of TLRs, would be necessary in polyclonal T cells in order for these cells to undergo LIP. In the same way, one could hypothesize that MyD88$^{-/-}$ Tregs

would be impaired in their ability to suppress polyclonal T cell LIP. Our initial acute LIP experiment suggested MyD88-deficient Tregs were competent to suppress spontaneous CD8 T cell proliferation (Fig. 4-5). What remained unclear, however, was whether APC recognition of TLR signaling was necessary for induction of suppression at peripheral tissue sites colonized by microflora. To answer this question we generated MyD88/RAG-1 double-deficient mice to use as recipients in an LIP-induced colitis experiment. We hypothesized that TLR ligation in recipient APC would be necessary for full activation of potentially pathogenic conventional CD4 T cells transferred, regardless of their own capacity for MyD88 signaling. Our results suggested MyD88 expression and TLR recognition in the host was responsible for much of the T cell expansion and IFN-γ expression that characterizes this model (Fig. 4-6B). This finding is consistent with a recent study suggesting MyD88 expression on intestinal epithelia and sensing of commensals is essential for maintenance of host homeostasis(186). To our surprise, however, transfer of WT CD4 T cells into MyD88-deficient hosts resulted in the greatest amount of leukocytic (white blood cell) infiltration and loss of host body weight typical of colitis induction (Fig. 4-6C). Even more surprising was the fact that MyD88 deficiency in the transferred conventional cells, regardless of host, protected the mice against colitic weight loss (Fig. 4-6A). Overall, these results suggest *direct* TLR recognition and activation of an inflammatory program in conventional CD4 T cells contributes significantly to leukocyte recruitment and inflammation of the colon. In the case where MyD88-deficient conventional cells were transferred into MyD88-deficient hosts, our results suggest insufficient activation and differentiation of the conventional

population protected recipients from colitis. Though untested, we cannot rule out up-regulation of Foxp3 expression in conventional cells in this case.

Conclusions and Future Directions

Following initiation of the studies described above, Shimon Sakaguchi and colleagues published a seminal manuscript in *Science* outlining the role CTLA-4 expression on Foxp3^{+} cells, specifically, plays in Treg peripheral homeostasis and suppression (187). Results presented suggest CTLA-4 expressed on the surface of Tregs actively down-regulates expression of co-stimulatory B7 molecules on the surface of interacting DC. Based on these results, CTLA-4 expression is not absolutely required for Treg development or survival, but plays an essential part in suppression of tumor immunity and the fulminate, systemic autoimmunity documented in Treg-deficient mouse models. Outstanding questions remain, however, regarding the roles of IDO and IL-10 expression in the presented model of Treg suppression. Specifically, the question of whether CTLA-4 expression on Tregs is necessary for not only B7 downregulation, but also IDO and/or IL-10 expression and adoption of an 'immunoregulatory' phenotype by DC was not addressed but warrants study. In an attempt to address this question, Sojka et al. assessed Treg-mediated suppression of acute (2 weeks) conventional T cell LIP in hosts treated with an IDO inhibitor (1-methyl-[D]-tryptophan or D-1MT). Results suggest Treg control of LIP is IDO-independent. An outstanding, albeit difficult, question not addressed in these studies is whether there is a role for concomitant IL-10 induction in these IDO inhibitor-treated DC (188).

An even more intriguing, and clinically relevant, question than the one posed above is whether thymic-derived Treg CTLA-4 ligation of B7 molecules on DC results in induction of adaptive Tregs. The Sakaguchi conditional knock-out mice were bred to express the fluorescent marker eGFP upon activation of the recombinase that deleted CTLA-4 expression (i.e. Cre). To address this question, one could envision an experiment comparing the induction of IL-10 expression in Foxp3- CD4 T cells (Tr1 or inducible Tregs) in conditional knock-out or WT CTLA-4 mice bred to a background conducive to organ specific inflammation (e.g. non-obese diabetic (NOD) mice) that would also allow *in situ* tracking of eGFP+ cells.

Competition with conventional cells for growth factors (e.g. IL-2 and IL-15)

Tregs share many traits with conventional T cells, such as antigen-specificity, co-stimulatory requirements (CD28 signaling), and responsiveness to γc cytokines (e.g. IL-2 and IL-15). They have low expression of the IL-7 receptor α chain, however, and do not compete for this essential homeostatic cytokine with conventional T cells during LIP (189). These cells are anergic due in part to direct inhibition of NFAT and NFkB activity by Foxp3, rendering them unable to produce cytokines such as IL-2 (190). However, expression of the high affinity IL-2 receptor on these cells enables them to consume this cytokine. The dependence of Treg homeostasis on T cell-derived IL-2 exemplifies the paradoxical role this cytokine plays in both immunity and peripheral self-tolerance.

Antibody-mediated neutralization of IL-2 in normal mice reduces the number of Tregs and results in autoimmunity akin to the *scurfy* mouse model (191). Likewise, IL-2- and IL-2Rβ-deficient mice succumb to lymphadenopathy and autoimmune colitis within the first few weeks of life (154). Competition for growth factors such as IL-2, therefore, is one of several potential mechanisms of suppression employed by Tregs. Interestingly, only one study has been published in recent years demonstrating this phenomenon (74). Treg consumption of IL-2 and concomitant deprivation of conventional cells was demonstrated *in vitro*. As a complement, *in vivo* data suggested colitogenic T cells deficient for the B cell lymphoma 2–interacting (Bcl2-interacting) mediator **(**Bim), a downstream target of IL-2 signaling, were resistant to Treg-mediated suppression. IL-15 is produced and presented by bone-marrow-derived innate cells to innate natural killer (NK), intraepithelial lymphocytes (IELs), and memory CD8 T cells in the context of intracellular bacterial infections and autoimmune inflammation (192). Suppression of the TGF-β-SMAD3 signaling pathway by IL-15 has been implicated in the promotion of celiac disease (CD), and recently published data from a lab at INSERM suggests IL-15 impairs Treg-mediated suppression by acting directly on conventional T cells, making them resistant (193, 194). This data corroborates that suggesting IL-15 independence of peripheral Tregs, despite sensitivity in the thymus in the absence of IL-2 (195, 196).

Results

We began studying the influence of IL-2 on Treg-mediated suppression of conventional cell LIP with a simple experiment involving transfer of WT Tregs into lymphopenic, IL-2-deficient hosts. As expected, we detected some defect in expansion of Tregs in absence of IL-2 (Fig. 4-7A). This dependence on IL-2 for expansion was specific to regulatory T cells, however, in that expansion of IL-2-deficient CD8 T cells in an IL-2-deficient host is enhanced in comparison to expansion of IL-2-sufficient CD8 T cells in an IL-2-deficient host (Fig. 4-7B). Although expansion is enhanced, the IL-2-deficient cells have lower levels of the TCR activation rheostat CD5 and the IL-2 receptor β chain (CD122), and they do not express the cytotoxic molecule granzyme-B (GZ-B), implying defect in function. Cells deficient for the apoptosis activator Bim are reportedly protected from forms of AICD involving inactivation of the pro-survival protein Bcl-2 (197). As mentioned above, Bim-deficient T cells are reportedly insensitive to Treg-mediated suppression. We wanted to test our hypothesis that Tregs compete for IL-2 as a means of suppression by investigating suppression of Bim-deficient T cells by Tregs during acute LIP. John Wherry and colleagues recently published an article in Immunity outlining the molecular signature of CD8 T cell exhaustion during chronic infection (198). Conventional T cells undergoing LIP up-regulate expression of the antigen experience marker CD44 and exhibit an effector phenotype. Assuming the CD8 T cell phenotype undergoing spontaneous LIP driven by 'chronic' commensal antigen stimulation would resemble that of exhausted, viral-specific CD8 T cells in immunoreplete mice, we analyzed WT and Bim-deficient T

cells post-LIP for expression of CFSE dilution and the senescence/effector cell marker killer cell lectin-like receptor G1 (KLRG-1) (198). Expression of KLRG-1 did indeed mark the most highly divided cells. It also showed utility as a marker for targets of Treg suppression (Fig. 4-8A). In this respect, and contrary to what has been reported, Tregs appeared to effectively suppress acute $Bim^{-/-}$ T cell LIP (Fig. 4-8A and B).

Evidence published initially by Surh and Sprent in 2007 and by several other researchers since suggests natural Tregs may mediated suppression of T cell proliferation/differentiation in the periphery through competition for IL-2. First, Treg-mediated suppression of T cell LIP appears to be preferential to fast-dividing cells (as measured by CFSE dilution), which are thought to be the most highly IL-2/15-sensitive fraction of proliferating CD8 T cells. Second, although naïve TCR transgenic OT-I CD8 T cells are known to be relatively insensitive to IL-2 (in comparison to IL-15) in that they have little surface expression of the IL-2 receptor α chain (CD25), following activation OT-I cells are known to up-regulate CD25 - increasing their sensitivity to IL-2 (199). As a means of generating IL-2-deficient mice for use in adoptive transfer experiments, we bred mice on an $IL\text{-}2^{+/-}$ background to OT-I $RAG\text{-}2^{-/-}$ mice. The advantage of using $IL\text{-}2^{-/-}$ OT-I $RAG^{-/-}$ cells for transfer into IL-2-deficient, lymphopenic mice over receptor-deficient OT-I cells, is that we may assess Treg-mediated suppression when IL-2 is lacking in either responders or recipients singly or together. The model we've proposed leaves cytokine responsiveness of the OT-I cells intact, which we believe to be essential for normal development. Before using $IL\text{-}2^{-/-}$ OT-I $RAG^{-/-}$ mice as donors for adoptive transfer experiments, we confirmed their phenotype by surface staining for effector and effector/memory markers KLRG-1 and

CD122, respectively (Fig. 4-9A). Upon transfer of IL-2$^{-/-}$ OT-I CD8 T cells into either WT or IL-2-deficient lymphopenic hosts we observed some defect in expansion in IL-2-deficient hosts, suggesting some role for recipient IL-2 in driving LIP. Unexpectedly, however, regulatory T cells were able to suppress the function of IL-2$^{-/-}$ OT-I CD8 T cells in IL-2-deficient hosts as measured by GZ-B expression (Fig. 4-9B). Perhaps even more surprising, expansion in the presence of Tregs actually increased the number of IL-2-deficient OT-Is detected in the LN (Fig. 4-9C).

We acknowledge the caveat that IL-2$^{-/-}$ OT-Is remain sensitive to IL-15 signaling. Based on reported relative insensitivity of CD8 T cell spontaneous LIP on IL-7 signaling, the results presented above, and the fact that Treg IL-2 requirements for LIP are not absolute (Fig. 4-7), one could hypothesize that GZ-B expression and unimpaired Treg-mediated suppression of IL-2-deficient OT-1s undergoing LIP results from compensation by IL-15. To address this caveat, we did a final experiment using IL-15$^{-/-}$ RAG$^{-/-}$ animals as recipients for WT polyclonal or OT-I RAG$^{-/-}$ CD8 T cells and assessed Treg suppression of LIP. Our results suggest unimpaired Treg-mediated suppression of both polyclonal and TCR transgenic OT-I CD8 T cells undergoing LIP in IL-15-deficient hosts (Fig. 4-10).

Conclusions and Future Directions

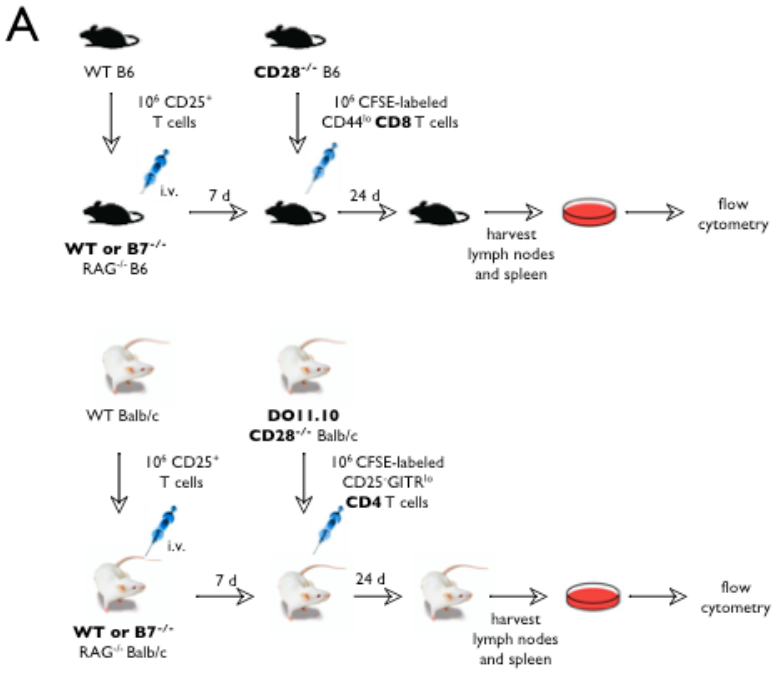
Results presented above suggest IL-2$^{-/-}$ OT-I CD8 T cells undergoing LIP develop an altered effector/memory phenotype. An open question is whether they are better or worse at clearing an infection. Usharauli and Kamala published a paper in

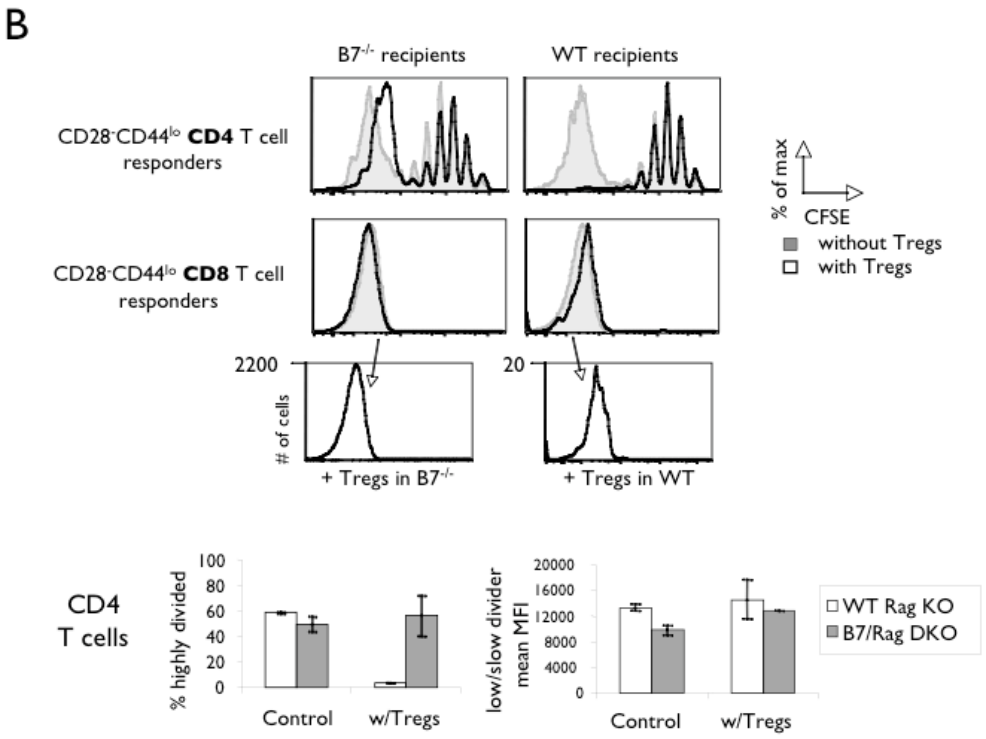
2008 focused on *in vitro* data suggesting a negative correlation between IL-2 production by OT-I T cells and cytotoxic potential as measured by GZ-B and the apoptosis-inducing ligand FasL (200). This is a 'hallmark' of central memory phenotype cells (as opposed to effector memory). When transferred into a normal host that is subsequently infected, these cells proliferated robustly and showed similar GZ-B expression to true 'effector phenotype' cells. The hyperproliferation potential of IL-2-deficient CD8 T cells in a lymphopenic host suggests IL-$2^{-/-}$ OT-I T cells would be better at cytotoxicity than wild-type cells. However, their ability to make GZ-B directly *ex-vivo* is impaired following LIP. This instead suggests they may be less cytotoxic, and recipients therefore more susceptible, if transferred without 'helper' cells (i.e. IL-2-competent CD4 T cells). Would Treg suppression rescue their cytotoxic phenotype somehow? Perhaps by preventing proliferation and antigen-induced exhaustion?

Listeria monocytogenes (LM) is an acute infection and some published data suggests IL-2-competency in T cells is important, primarily, in late stages of chronic infection. Perhaps the optimal infection model to answer the questions posed above would not be LM, but lymphocytic choriomeningitis virus (LCMV) clone 13, known to establish chronic infection in mice (201). The genome size of LCMV prohibits insertion of OVA (a large protein), however. *Leishmania major*, a protozoan parasite, is another chronic infection model used to assess immune fitness. The *Leishmania* genome is large enough to allow insertion of the entire OVA protein, however this model is complex in that both T_H1 CD4 T cells and CD8+ CTLs are important for resistance. A recent article published in the *Journal of Experimental Medicine* suggests infection of lymphopenic mice (e.g. $RAG^{-/-}$) with LM results in 'chronic' infection that eventually kills the host,

albeit with delayed kinetics (202). We know that precursor frequency of antigen-specific cells, especially in regard to OT-I cells and LM, dictate the quality of memory established in infected animals, based on work published by Harty and colleagues (203). With this information at hand, we can propose a series of experiments to further elucidate the pleiotropic effects of IL-2 in CD8 and regulatory CD4 T cell homeostasis. We could first compare measures of CD8 T cell exhaustion following direct LM infection of IL-$2^{-/-}$ and WT OT-I $RAG^{-/-}$ animals. An alternative to this experiment could also involve adoptive transfer of naïve IL-$2^{-/-}$ or WT OT-I $RAG^{-/-}$ into host WT mice at physiological numbers (~200/mouse) followed by LM infection and measures of exhaustion (e.g. KLRG-1). We could then follow-up this experiment with functional measures using a technique we discussed in Chapter 3 of this thesis. That is: adoptive transfer and subsequent LIP of either IL-$2^{-/-}$ or WT OT-I $RAG^{-/-}$ transferred into either IL-$2^{-/-}$ or WT $RAG^{-/-}$ hosts, followed by isolation of memory cells, secondary transfer (at physiological numbers ~200/mouse) into WT hosts, and infection with LM. This experiment would be complemented well by established chronic LM infection in $RAG^{-/-}$ hosts followed by transfer of physiological numbers of IL-$2^{-/-}$ or WT OT-I $RAG^{-/-}$ and measures of bacterial clearance. Finally, we could determine the potential for Tregs to alter the outcome of each experiment by both adoptive transfer of cells and treatment with antibodies to IL-2.

FIGURE 4-1. CD28$^{-/-}$ T cells undergoing LIP in a B7$^{-/-}$ host are insensitive to Treg-mediated suppression. *A*. The basic experimental protocol involved pre-filling of the Treg niche in lymphopenic recipients via adoptive transfer of CFSE-labeled or unlabeled Tregs (CD25+ T cells). *B*. Plots represent lymph node FACS from one representative animals per condition. Responder T cells were gated using staining for the Thy-1 congenic marker and either CD4 or CD8. Bar graphs represent the mean ± SD of two animals. MFI = mean fluoresecence intensity. *C*. Each plot depicts CFSE dilution of Tregs in the lymph nodes from one representative animal harvested on Day 0. Each bar represents the mean ± SD of two animals.




B
B7-/- recipients
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CD28-CD44lo CD4 T cell responders
CD28-CD44lo CD8 T cell responders
% of max
CFSE
without Tregs
with Tregs
2200
20
# of cells
+ Tregs in B7-/-
+ Tregs in WT
CD4 T cells
% highly divided
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WT Rag KO
B7/Rag DKO

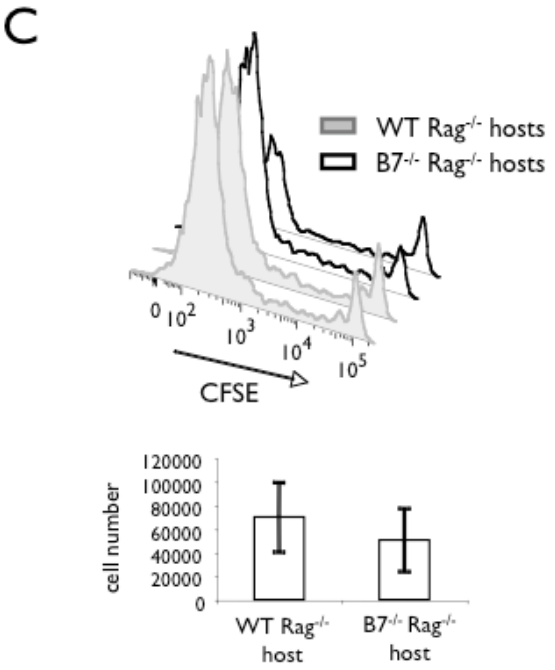

C
WT Rag-/- hosts
B7-/- Rag-/- hosts
CFSE
cell number
WT Rag-/- host
B7-/- Rag-/- host

FIGURE 4-2. Treg-mediated suppression of spontaneous LIP is IDO-independent. CFSE-labeled or unlabeled CD25+ T cells were injected via tail vein into B6 hosts on Day -7 (5 x 10^5 per recipient). Recipients of CFSE-labeled Tregs were harvested for FACS analysis on Day 0. Approximately 2 x 10^5 polyclonal $CD44^{lo}CD4$ T cells were CFSE-labeled and injected into the remaining, as well as a fresh set of, recipients. All animals were harvested on Day 15. Responder T cells were gated using staining for Thy-1 congenic marker and CD4. *A*. Representative CFSE profiles of responding T cells in the lymph nodes are from single animals. *B*. Bar graphs represent the mean ± SD of three animals per condition.

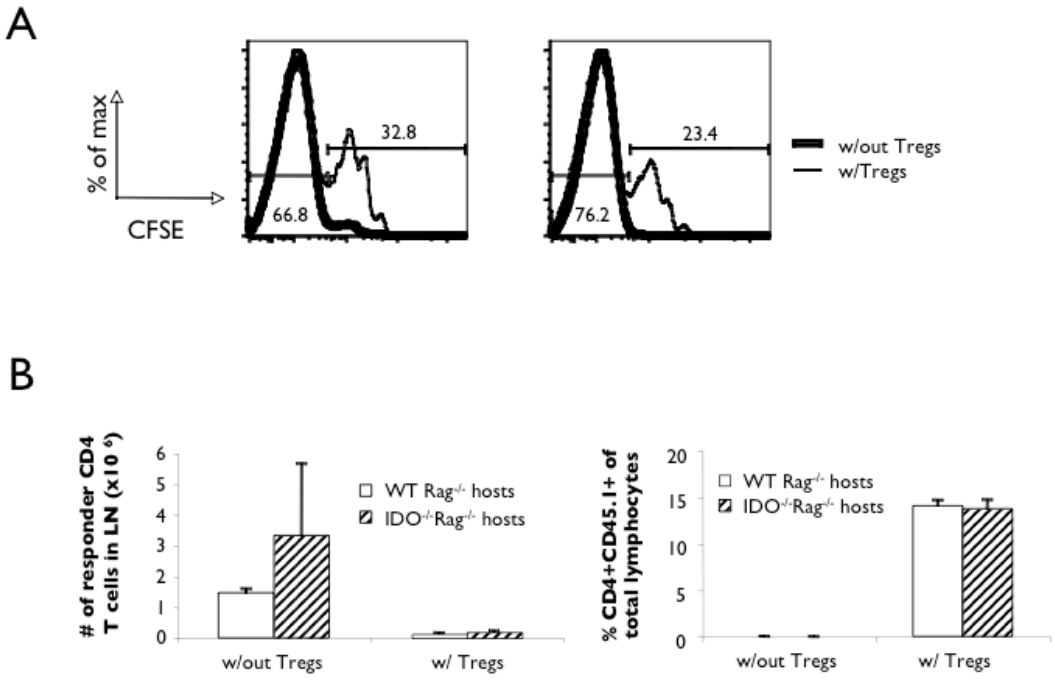
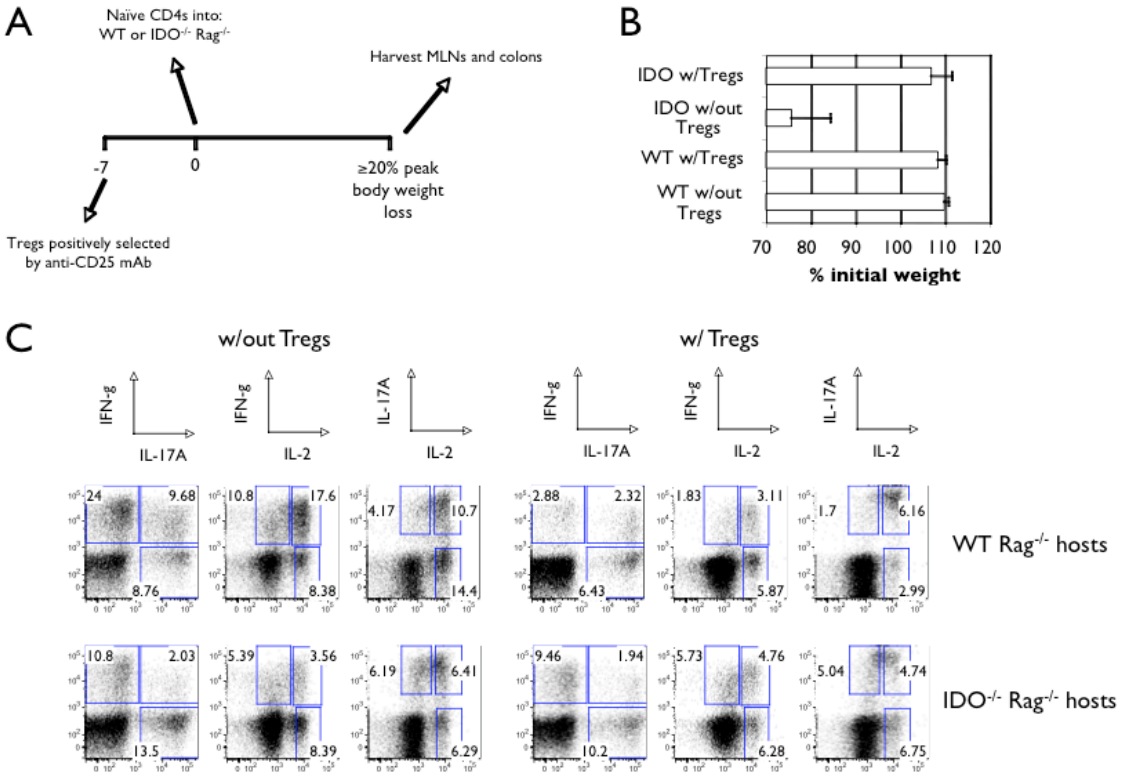


FIGURE 4-3. Protection from adoptive transfer-induced colitis is partially IDO-dependent. *A*. Similar to Figure 1A, the basic experimental protocol involved pre-filling of the Treg niche in some lymphopenic recipients via adoptive transfer on Day -7 of 5 x 10^5 Tregs (CD25+ T cells). Approximately 1 x 10^5 polyclonal $CD44^{lo}CD4$ T cells were CFSE-labeled and injected into the same recipients as well as fresh recipient mice on Day 0. *B*. Animals were sacrificed after animals in the 'IDO w/out Tregs' group lost ≥ 10% initial body weight. Responder T cells were incubated with PMA, ionomycin, and Brefeldin A before staining for FACS. *C*. Representative FACS of responding T cells in the mesenteric lymph node (MLN) are from single animals. *B and D*. Bar graphs represent the mean ± SEM of three animals per condition.



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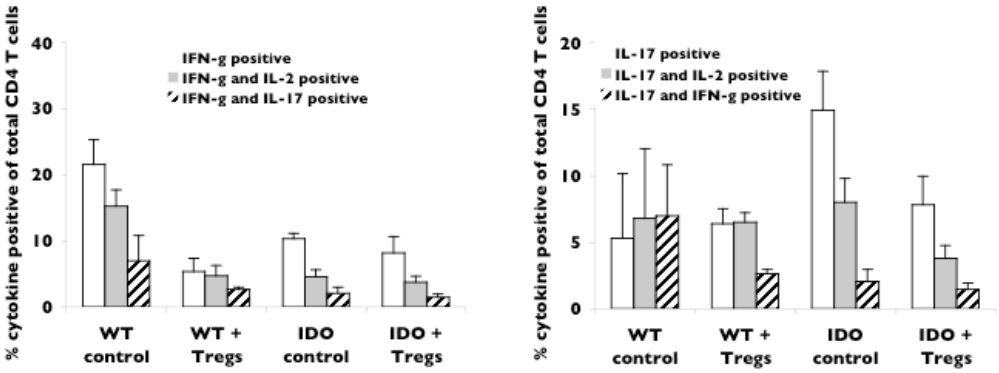
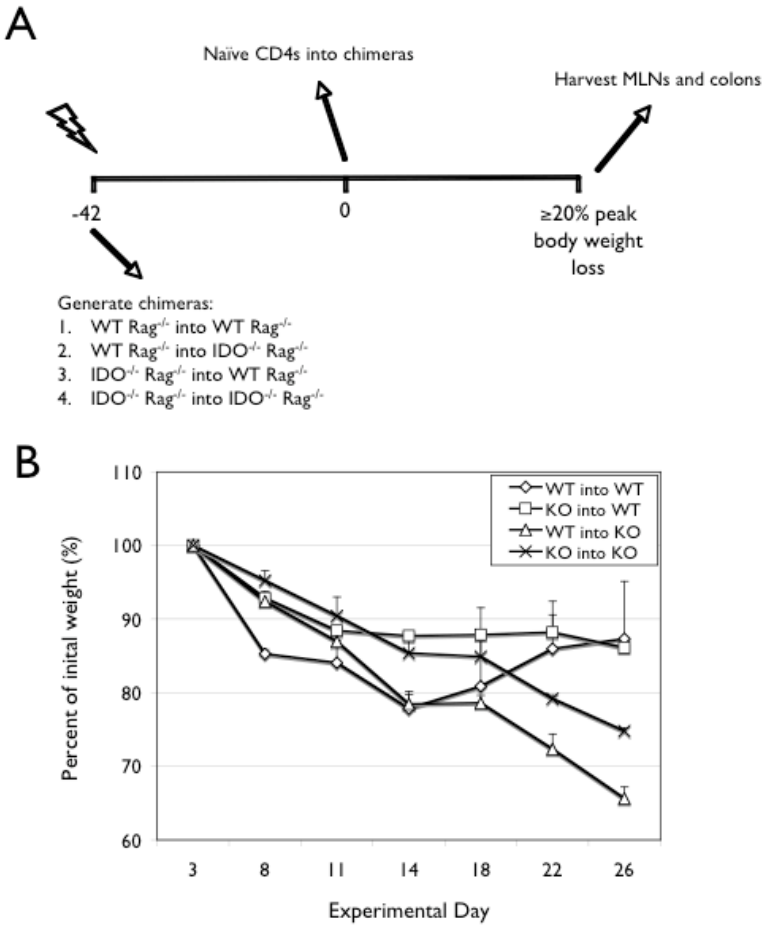

% cytokine positive of total CD4 T cells
40
30
20
10
0
IFN-g positive
IFN-g and IL-2 positive
IFN-g and IL-17 positive
WT control
WT + Tregs
IDO control
IDO + Tregs
% cytokine positive of total CD4 T cells
20
15
10
5
0
IL-17 positive
IL-17 and IL-2 positive
IL-17 and IFN-g positive
WT control
WT + Tregs
IDO control
IDO + Tregs

FIGURE 4-4. IDO expression in radio-resistant cells protects from colitis via Treg-induction in the colon. *A and B*. Chimeric animals were prepared as depicted using transfer of 10 million cells from RAG-/- donor bone marrow into RAG-/- hosts irradiated with two doses of 400 rads separated by 3-4 hours. 1 x 10^5 naïve ($CD44^{lo}$) CD4 T cells were transferred 6 weeks later and all animals harvested on Day 27. *C*. Bar graphs represent the mean reference bead-based counts of CD4+Foxp3+ cells ± SD of four animals per condition.



C

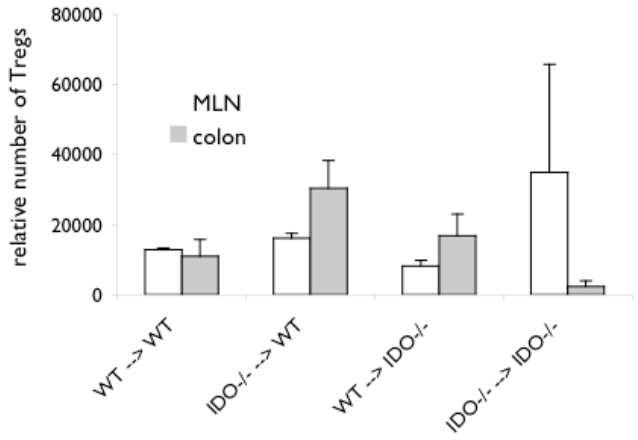

relative number of Tregs
80000
60000
40000
20000
0
MLN
colon
WT --> WT
IDO-/- --> WT
WT --> IDO-/-
IDO-/- --> IDO-/-

FIGURE 4-5. MyD88-/- Tregs are competent suppressors of polyclonal CD8 T cells spontaneous LIP. 3 x 10^6 MyD88-/- or wild-type (WT) CD25+ T cells were injected via tail vein into each RAG-/- B6 host on Day -7. Approximately 5 x 10^6 polyclonal $CD44^{lo}$CD8 T cells were CFSE-labeled and injected all recipients, as well as a fresh set of RAG-/- animals. All animals were harvested on Day 10. Responder T cells were gated using staining for Thy-1 congenic marker and CD8. Plots represent lymph node FACS from one representative of two animals per condition.

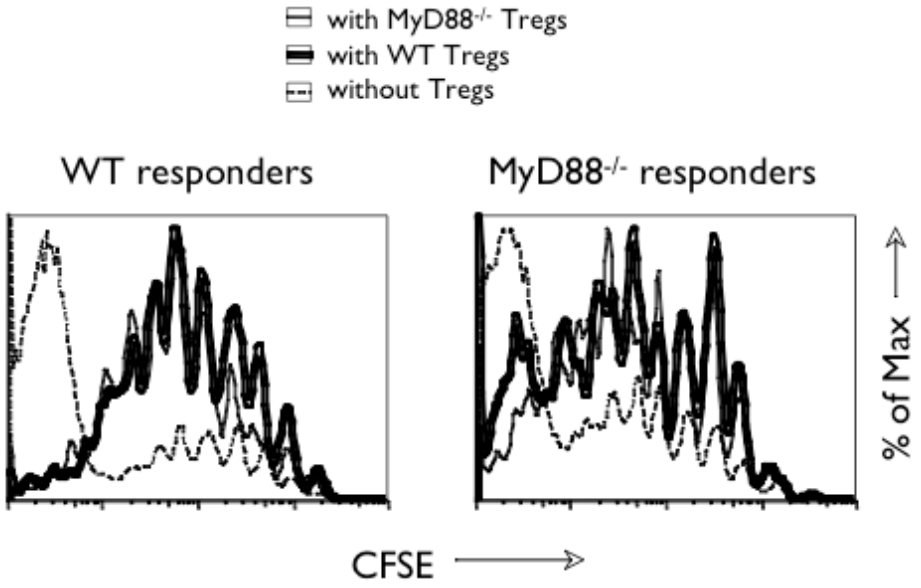


FIGURE 4-6. Loss of TLR signaling in responder CD4 T cells confers some protection from adoptive transfer-induced colitis without preventing activation and differentiation to an effector phenotype. *A*. Approximately 1 x 10^5 MyD88-/- (KO) or WT polyclonal $CD44^{lo}$CD4 T cells were injected into MyD88-/- RAG-/- or WT RAG-/- recipients on Day 0. B. Animals were sacrificed 6 weeks later. *B*. Responder T cells were incubated with PMA, ionomycin, and Brefeldin A before staining for FACS and bead count (relative) enumeration. Bar graphs represent the mean ± SD of gated CD3+CD4+ MLN cells from four animals per condition. *C*. Histologic scores were determined on sections of distal colons while blinded to the identity of the experimental groups.

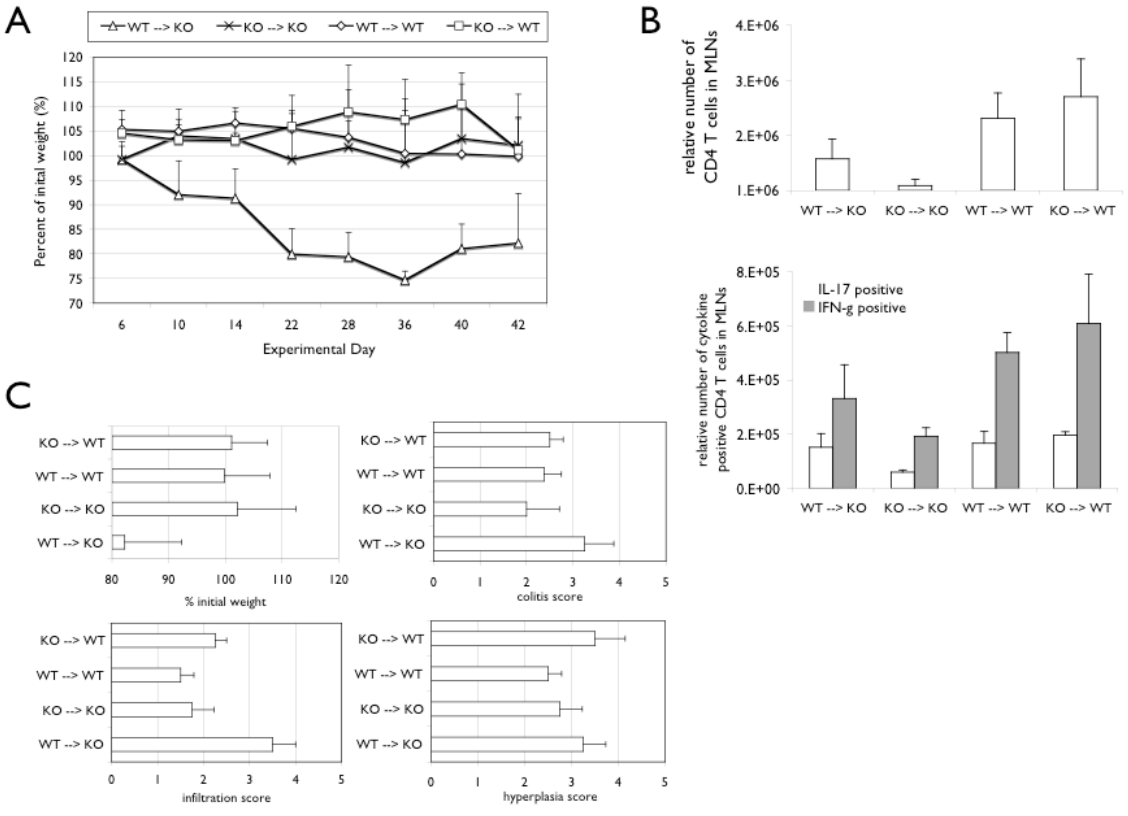
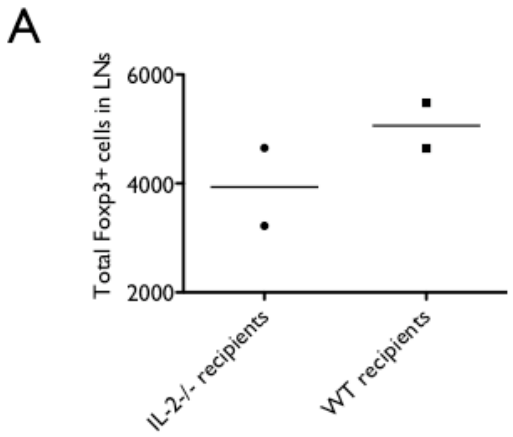


FIGURE 4-7. Tregs are partially dependent on IL-2 for LIP, whereas IL-2-/- CD8 T cells exhibit IL-2-insensitive hyperproliferation in WT lymphopenic hosts. *A*. 6 x 10^5 CFSE-labeled CD25+ T cells were injected via tail vein into IL-2-/- or WT B6 hosts on Day -7. Lymph nodes were harvested for FACS analysis 7 days later. Graphed data represents the mean number of Foxp3+ in LNs from 2 animals per condition. *B*. Approximately 2 x 10^5 polyclonal $CD44^{lo}CD8$ T cells from either IL-2-/- or WT B6 donors were CFSE-labeled and injected into WT RAG-/- recipients on Day 0. LNs were harvested from all animals 10 days later. Responder T cells were gated using staining for Thy-1 congenic marker and CD8. CFSE profiles depict one representive animal per condition. FACS profiles of responding T cells in the lymph nodes represent two animals per condition.



B

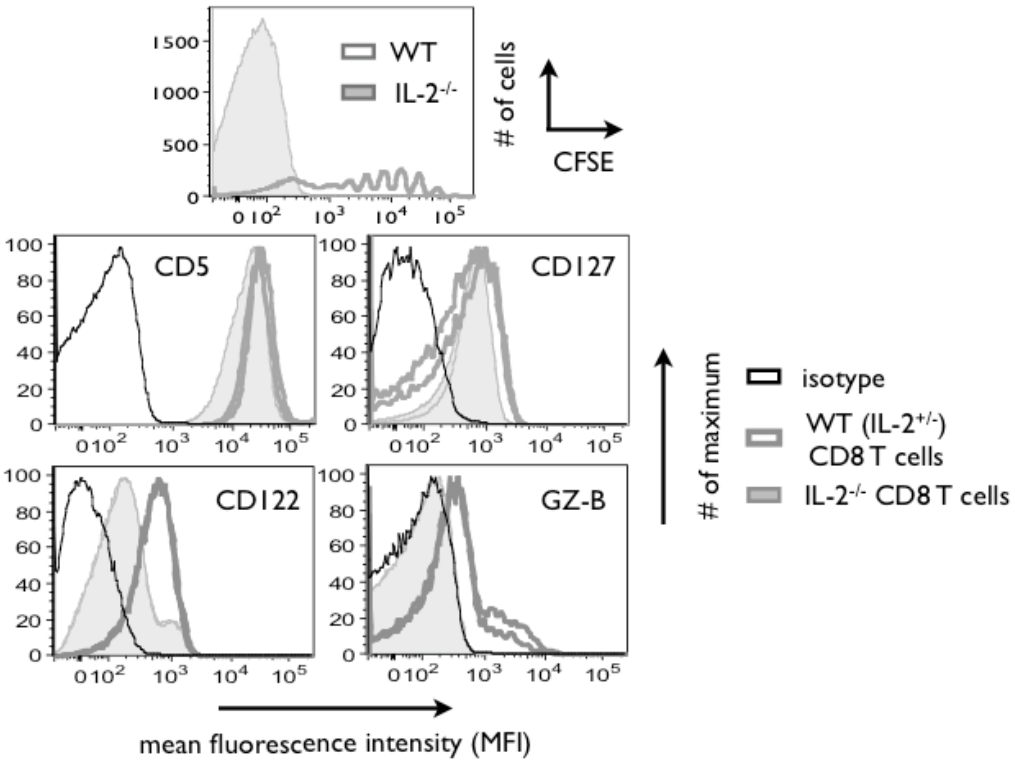

WT
IL-2-/-
# of cells
CFSE
CD5
CD127
CD122
GZ-B
mean fluorescence intensity (MFI)
# of maximum
isotype
WT (IL-2+/-) CD8 T cells
IL-2-/- CD8 T cells

FIGURE 4-8. Bim-/- T cells are sensitive to suppression of spontaneous LIP by Tregs. 1 x 10^6 CD25+ T cells were injected via tail vein into each RAG-/- B6 host on Day -8. A 1:1 mix of 1 x 10^6 total Bim-/- and WT $CD44^{lo}$CD8 or CD4 T cells were CFSE-labeled and injected into all recipients, as well as a fresh set of RAG-/- animals. Lymph nodes from all animals were harvested on Day 10. Responder T cells were gated using staining for CD3, CD4 or CD8, CD45.2 and a Thy-1 congenic marker (Thy1.1+ = WT; Thy1.1- = Bim-/-). *A*. Plots represent FACS from one representative of two animals per condition. *B*. Bar graphs represent the mean ± SD of two animals per condition.

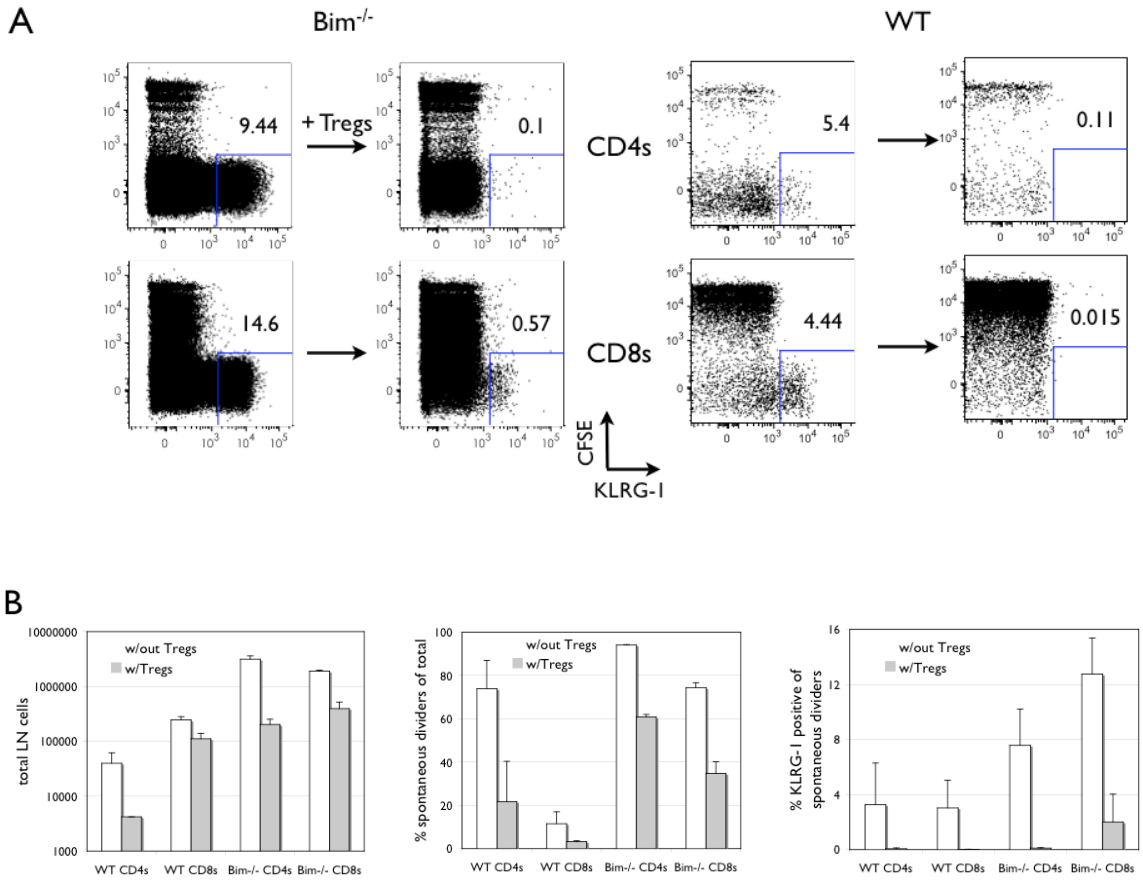


FIGURE 4-9. IL-2-/- CD8 T cells undergoing LIP in an IL-2-/- host are partially defective for effector function but sensitive to Treg-mediated suppression. *A*. Splenocytes from an IL-2-/- (KO) OT-I RAG-/- mouse express lower levels of traditional 'memory' markers CD44 and CD122, and have comparatively fewer KLRG-1-expressing cells in the steady state. *B*. 1 x 10^6 CD25+ T cells were injected via tail vein into IL-2-/- or WT B6 hosts on Day -7. 1 x 10^5 IL-2-/- OT-I RAG-/- cells were CFSE-labeled and injected into the same, as well as a fresh set of RAG-/-, recipients on Day 0. LNs were harvested from all animals 10 days later for intracellular staining for granzyme-B (GZ-B) and FACS analysis directly *ex vivo*. CFSE profiles depict both animals, one above the other, per condition. *C*. Bar graphs represent the mean ± SD of two animals per condition.

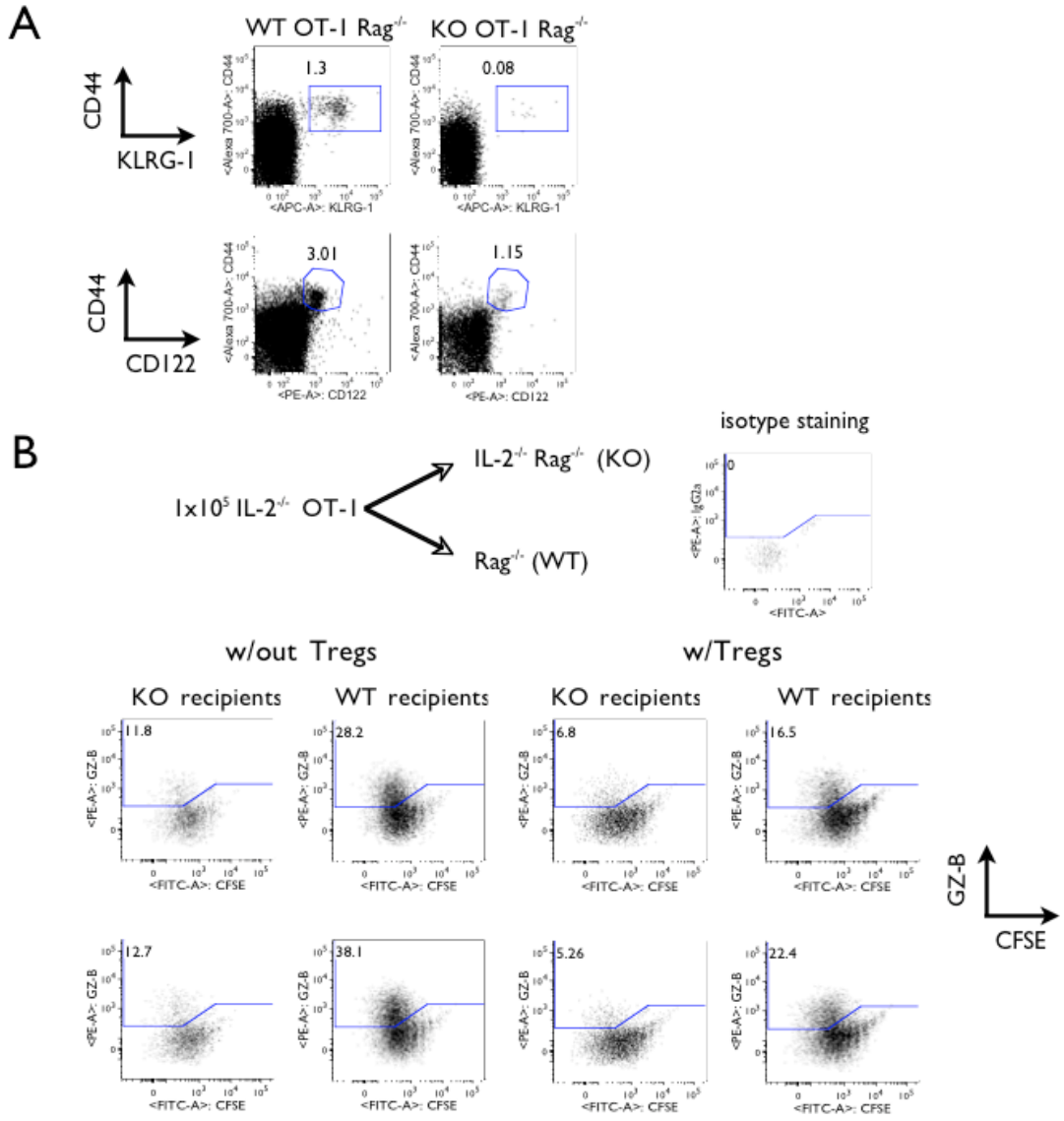
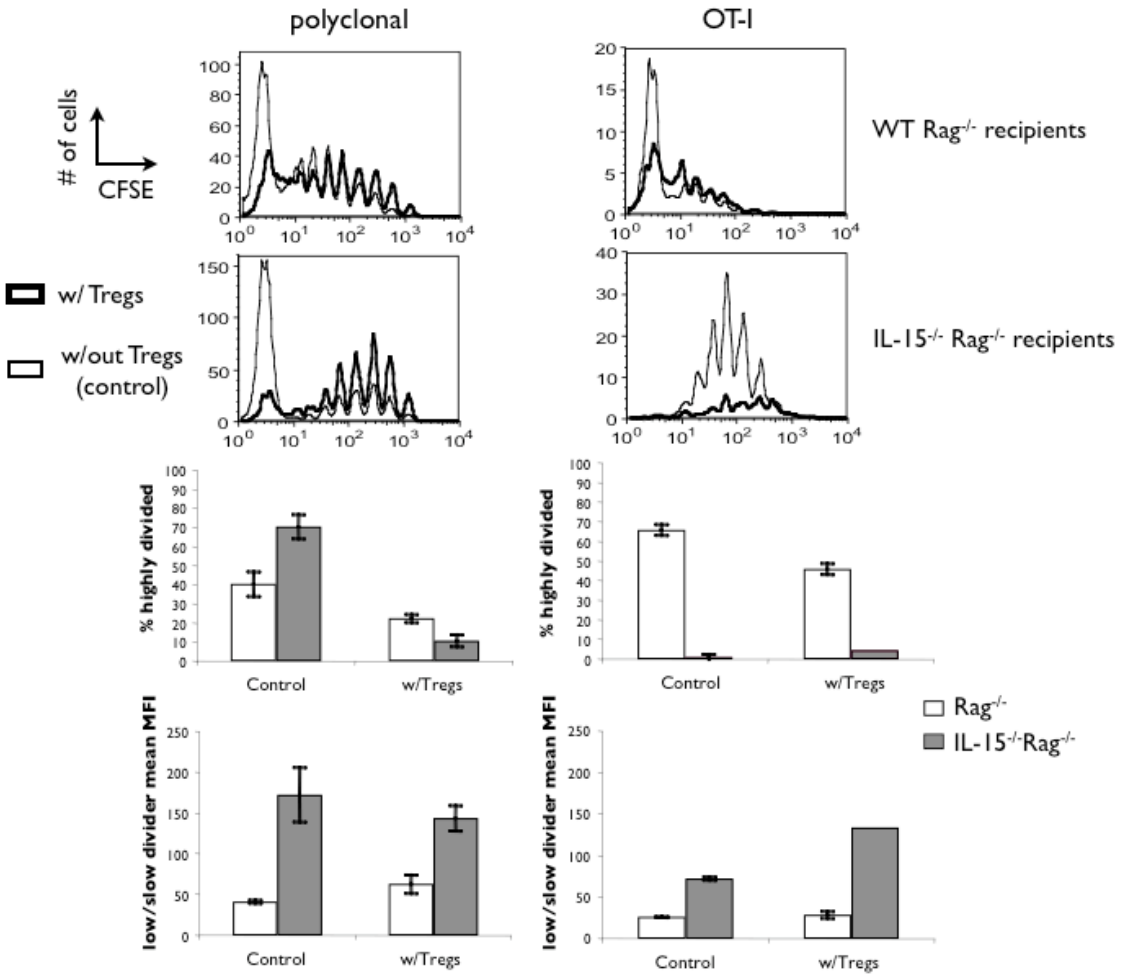

A
WT OT-1 Rag-/-
KO OT-1 Rag-/-
CD44
KLRG-1
1.3
0.08
<APC-A>: KLRG-1
CD44
CD122
3.01
1.15
<PE-A>: CD122
B
1x10^5 IL-2-/- OT-1
IL-2-/- Rag-/- (KO)
Rag-/- (WT)
isotype staining
<FITC-A>
w/out Tregs
w/Tregs
KO recipients
WT recipients
KO recipients
WT recipients
11.8
28.2
6.8
16.5
12.7
38.1
5.26
22.4
<PE-A>: GZ-B
<FITC-A>: CFSE
GZ-B
CFSE
C
IL-2-/- OT-1 cell number in LNs (relative to total lymphocytes)
16000
12000
8000
4000
0
KO + TR
KO
WT + TR
WT
Recipients

FIGURE 4-10. Polyclonal and TCR Tg OT-I cells have different IL-15 requirements for spontaneous LIP but are both sensitive to Treg-mediated suppression in IL-15-/- hosts. 2 x 10^6 CD25+ T cells were injected via tail vein into IL-15-/- or WT RAG-/- B6 hosts on Day -7. 5 x 10^5 $CD44^{lo}$ polyclonal CD8 T cells or 8 x 10^4 OT-I RAG-/- cells were CFSE-labeled and injected into the same, as well as a fresh set of RAG-/-, recipients on Day 0. LNs were harvested from all animals 10 days later for FACS analysis. CFSE profiles depict one representative animal per condition. Bar graphs represent the mean ± SD of two animals per condition.



Chapter 5

Discussion

Future directions for this thesis research

Assessing and improving T cell structural and functional diversity

Although we touch briefly on the topic of TCR affinity in reference to tetramer enrichment data presented in chapter 3 of this thesis, a more thorough assessment of TCR affinity would allow definitive conclusions to be drawn regarding fitness of both the antigen-specific CD4 T cell response and the quality of help provided to antigen-specific CD8 T cells. The finding that CDR3 (Jβ) spectratyping post-LIP in the absence of Tregs revealed holes in the repertoire not visible by spectratyping at the level of Vβ supports recent work published by the Kappler group suggesting the somatically generated CDR3 loops confer structural p-MHC-binding specificity. The Kappler group found that the specificity for p-MHC is distinct from that for MHC alone, as determined by the germ line-encoded TCR CDR1 and CDR2 loops (204). The implications of this finding are that single amino acid differences in CDR3 loop sequence may dictate whether a T cell has stronger self- or foreign-reactivity. A simple and testable hypothesis based on data presented here would be that infectious antigen-specific T cell clones enriched post-LIP in the presence of Tregs exhibit a pattern of CDR3 sequence consistent with strong foreign-reactivity. As discussed in Chapter 2, T cells undergoing LIP that are self antigen-specific and/or cross-reactive to commensal antigens are contained in the 'spontaneous' fraction. One would expect the percentage of total and absolute number of these would, in contrast to foreign antigen-specific clones, be diminished in the presence of Tregs. Considering the antigen-specific data presented in

Chapter 3, one could envision significant, complementary data using MHC tetramers to enrich cells specific for a highly expressed cross-reactive commensal antigen (e.g. peptide(s) from a select species of *Helicobacter*). In this instance, we predict these clones would have CDR3 sequences consistent with self-reactivity.

Questions also remain regarding whether antigen-specific T cells expanded in a lymphopenic host are phenotypically functional. Although controversial, one T cell differentiation paradigm suggests a single naïve cell is capable of giving rise to diverse effector and memory lineages in response to infectious stimuli (173, 205). Data presented in Chapter 3 of this thesis suggests that a comparison between the few antigen-specific cells that remain following LIP in a host devoid of Tregs with hosts pre-loaded with Tregs is feasible. This could be accomplished through selection and transfer of tetramer-specific cells into a secondary host. As presented in chapter 3 supplementary figure 4, secondary transfer would allow assessment in and direct comparison to function in a normal, non-inflammatory host. A potential disadvantage to this technique would be masking of transferred cell activity by resident responders. We provided preliminary data using a logical alternative showing potential for resolution of this issue – that is, the TCR$\alpha^{-/-}$ mouse as host for T cell LIP. Using this lymphopenic model, further cell surface and intracellular (cytokine) phenotyping of cells in a reconstituted mouse could be performed.

Using cytokine therapy to improve fitness during T cell reconstitution

If a particular phenotype of CD4 and/or CD8 T cell is most beneficial, one could then explore mechanisms to drive that phenotype. For example, if expanded CD4 T cells producing the γc chain cytokine IL-21 provide optimal help to pathogen- or tumor-specific CD8 T cells, then perhaps delivery of IL-21 alone or cytokine/antibody complex during reconstitution would improve fitness (206-208). Clinical studies focused on CD8 T cell function in patients with melanoma and active HIV-1 infection suggest IL-21 as a promising therapeutic, however emerging studies in patients treated with the immunosuppressive drug Campath-1H suggest potential CD4 T cell-dependent autoimmunity may result from a combination of LIP and systemic IL-21 treatment (209-211).

Some utility for use of IL-7 in this capacity has also been shown recently in human patients refractory to standard treatments for cancer (212). After receiving treatment in this study subjects showed improved peripheral T cell diversity (both structural and functional) and T cell reactivity to weak antigens consistent with preferential expansion of naïve anti-tumor conventional cells over Tregs. Potential treatments with IL-7 as a means to improve T cell diversity in a lymphopenic host should be approached with caution, however, as self-reactivity can become pathological under certain circumstances. Epithelial cells of the mucosa are thought to contribute to development of colitis through provision of IL-7 to colitogenic CD4 T cells in both mice and humans (213). A recent mouse study published by Tak Mak and colleagues suggests a potential consequence of provision of IL-7 to a reconstituting host is CD4 T

cell help to self-specific, pathogenic CD8 T cells and induction of autoimmunity (214). As suggested by the hygiene hypothesis, commensal organisms play an essential role in establishing and maintaining a fit immune system. Though many autoimmune diseases are linked to polymorphisms of MHC class II, there appears to be a strong environmental component contributing to pathogenesis. In fact, a recent study focusing on pathogen influence in selection of SNPs (small nucleotide polymorphisms) in genes associated with human autoimmunity found significant influence of parasites on the IL-7 receptor (33).

IL-2 and IL-2/antibody complex therapy is already being tested in the clinic in combination with other anti-tumor therapies. A report published in 2007 on the use of Daclizumab (anti-Tac or anti-IL-Rα) to treat leukemia suggested combined treatment with chemotherapy was effective (215). Careful study of the cytokine itself and components of its receptor has revealed subtle differences between Tregs and conventional T cells in sensitivity and dependence on IL-2 for survival and function. In a landmark study published in 2006, Charlie Surh and colleagues outline the subtleties of IL-2 binding to its low (β and γc) and high affinity (α, β, and γc) receptors and provide a framework for targeted T cell therapy (216). Two subsequent studies focused on the role of IL-2 signaling in Tregs suggest heightened sensitivity to this cytokine and induction of anti-inflammatory suppression confers protection against both organ-specific and fulminate autoimmunity in mice (217, 218).

The low affinity receptor for IL-2 is shared by IL-15. Unlike IL-2, IL-15 is presented preferentially to memory CD8 T cells. Waldmann and colleagues have found that combination treatment with APC-activating antibodies to CD40 and IL-15/receptor

α antibody complexes very effective for treatment of tumor-bearing mice (219). Transgenic/bioavailable expression of IL-15 has been reported to counteract IL-2-mediated AICD and promote proliferation of virus-specific memory CD8 T cells in the mouse (16). Though attractive as an anti-tumor therapy, this action of IL-15 comes at a price. That is, potential loss of both passive (AICD) and active (Treg-mediated) regulation of self-specific CD4 T cells and induction of autoimmunity.

Questions regarding Treg mechanisms of suppression

After more than ten years of careful research into Treg mechanisms of suppression, the field seems to have more open questions than available answers. Controversy stems in large part from the fact that no single mechanism applies to any specific pathology. In work presented here, we have taken advantage of the reductionist, adoptive transfer model to minimize confusion regarding source of Tregs. The Tregs used in this model are overwhelmingly thymic-derived. A natural next step in investigation that is also important for clinical applications, would be to assess the utility of adaptive/peripherally-induced Tregs in this reconstitution model. In a timely study published earlier this year, McLachlan and colleagues employ the same antigen-specific CD4 T cell detection methods used in chapter 3 of this thesis to generate and phenotype adaptive Tregs in the skin of peptide-immunized mice (220). These Tregs mediate suppression primarily through expression and secretion of IL-10. Whether or not these adaptive antigen-specific Tregs would function similarly to the thymic-derived

Tregs used in reconstitution experiments presented here, in regards to either structural or functional filling of T cell niches, warrants further study.

Our preliminary data presented in chapter 4 of this thesis and publications supporting the essential role of CTLA-4 expression on Tregs for prevention of autoimmunity in the lymphoreplete state suggest this protein also plays an essential role in suppression of spontaneous LIP and preservation of T cell diversity; most likely in combination with other mechanisms. Anti-inflammatory blockade of co-stimulation with CTLA-4Ig has shown great clinical application and has been approved by the FDA for use to treat rheumatoid arthritis. Emerging preclinical data on combination treatment with CTLA-4Ig and IDO-expressing DCs suggest even greater utility than treatment with CTLA-4Ig alone (221). In a recent *Immunity* review, Ethan Shevach reiterates the sentiment of several well-respected researchers in the field of Treg-mediated suppressive mechanisms: that the future of the field will involve study of multiple mechanisms working in tandem (at different or the same peripheral sites), sequentially, or stochastically, and will require increasingly sophisticated in vivo modeling techniques to advance (41).

Anti-pathogen T cell fitness in regards to pathogenic organism trophism

The question of whether T cell memory migration and pathogen trophism coincide is a long-debated and controversial one (222). An enormous amount of research focused on T cell homing to peripheral tissues and site specificity in expression of T cell growth factors (IL-2, -7, -15, -21) suggest distribution of memory cells to non-

lymphoid peripheral sites following pathogen clearance should be an important, focused goal of vaccination. Experimental data presented in chapter 3 of this thesis suggest preservation of foreign antigen specificity during T cell reconstitution is an important countermeasure to subsequent infection. However, the influence of infection preceding or concurrent with reconstitution warrants careful study, as this mimics several human conditions including re-emergence of malaria in patients treated with HAART (highly active anti-retroviral therapy for HIV) and the association of 'environmental triggers' (e.g. viral infections) with autoimmunity induced in response to low-affinity T cell cross-reactivity to microbial peptides (223).

The attenuated strain of LM used in experiments presented in chapter 3 of this thesis primarily infects the spleen, liver, and lung. However, as discussed in chapter 1, the natural route of *Listeria* infection is not intravenous but intestinal. The gut is likely the place where most adoptively transferred cells are activated (by self/cross-reactive commensal antigens), and questions regarding the ability of Tregs to balance anti-self and anti-pathogen responses during reconstitution in this unique environment are outstanding. It would be prudent and possibly even more significant to repeat the LM infection experiments presented in chapter 3 using a strain that is mutated to adhere to mouse gut epithelium (101). Questions of how 'fit' T cells emerging from LIP in the presence of Tregs are for combating a virulent infection (e.g. LM expressing HSV-1 immune epitopes), a chronic infection (e.g. LCMV), a heterologous infection (e.g. *Leishmania*) with different trophism, or even tumor challenge remain open (224).

The future of Treg adoptive therapy

There is published evidence to suggest CD4 and CD8 T cells respond differently to presented, peripheral self-antigens in the absence of immunoregulation, however the consequences of exploiting regulation for therapy to treat certain conditions is a relatively new field (225). Adoptive T cell therapy has shown some utility in combination with chemotherapy for the treatment of cancer. However, anti-inflammatory therapies that target T cells and antibody therapies for *in vivo* expansion of Tregs are not as effective and, in some cases, harmful (e.g. CD28 superagonist antibody TGN1412) (226). The field of adoptive Treg therapy using *ex vivo*-expanded cells likewise has been plagued by technical hurtles over the past few years that are just now being overcome. Some of the controversy in the field stems from the fundamental differences between disease treatment and disease prevention. The use of adoptive Treg therapy to prevent graft-versus-host-disease (GVHD) – a potential outcome of bone marrow and hematopoietic stem cell transplant (HSCT) – shows much promise for the future and is already being tested in the clinic (227). The benefits of adoptive Treg therapy to treat established autoimmunity are less clear, however. The isolation, expansion, and reinfusion of Tregs from an autoimmune patient poses a risk of exacerbating rather than treating the condition because the autoimmune microenvironment is inflammatory and could potentially convert regulatory cells back into an effector phenotype. On the other hand, it is easier to treat than predict onset of

disease. Techniques for in vitro expansion of antigen-specific Tregs for later reinfusion have advanced greatly in the last few years, in part due to emphasis on research related to the plasticity of T cell development, activation, and differentiation. Though issues with potency, purity, and selection post-expansion of the adoptive population continue, our knowledge base concerning requirements for *in vitro* conversion of conventional T cells and what biological or chemical mediators reinforce the regulatory phenotype has grown considerably in the last few years (228). The lack of a reliable Treg-exclusive T cell marker has hampered development of technologies for reversal of suppression that would be a valuable addition to some cancer therapies, however. Though GITR ligation, stimulation with IL-6, and OX-40 engagement of conventional T cells by DC are all reported to make them resistant to Treg-mediated suppression, the potential for inducing autoimmunity exists (41, 229, 230).

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